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Phosphorylation of the Human Papillomavirus Type 16 E1^E4 Protein

A thesis submitted to the University of London for the degree of Doctor of Philosophy

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Abstract

Human papillomaviruses (HPVs) are linked to over 99 % of cervical cancers and HPV16 is found in approximately half of all cases. The 16E1^E4 protein is abundantly expressed in productive infections but is downregulated or lost in cancers. The protein colocalises with and induces the collapse of cytokeratin networks, and in doing so, may facilitate virus release from cells. 16E1^E4 also causes cell cycle arrest by binding to and affecting the function of CDK1/cyclin B, which may enhance the virus' ability to use cellular DNA replication machinery to amplify its viral genome. 16E1^E4 has also been shown to colocalise with CDK2/cyclin A.

Phosphorylation of 1E1^E4 and 11E1^E4 has been shown *in vivo*. In this study, phosphorylation of 16E1^E4 was investigated. When 16E1^E4 is expressed in cell culture, a slower-migrating, phosphorylated form is seen after SDS-PAGE. 16E1^E4 is phosphorylated by CDK1, CDK2, p42MAPK, PKA and PKCα *in vitro*. p42MAPK *in vitro* phosphorylation results in a slower-migrating form of 16E1^E4 similar to that seen in cell culture. Mass spectrometry and mutagenesis was used to map some *in vitro* phosphorylation sites. Mutagenesis, isoelectric focusing and the use of kinase inhibitors, suggest that when 16E1^E4 is expressed in cell culture, serine 32 is phosphorylated by CDK1/2 and threonine 57 by p42/p44MAPK. Threonine 57 phosphorylation causes the observed change in migration with SDS-PAGE. A bacterially-expressed 16E1^E4 with threonine 57 mutated to aspartic acid to mimic phosphorylation, was shown to bind keratin more strongly than the unphosphorylated, wild-type 16E1^E4. Phosphorylation may therefore have a role in regulating 16E1^E4 activity *in vivo*. Coexpression of the HPV16 E5 protein was found to enhance threonine 57 phosphorylation by activating p42/44MAPK, suggesting another function for E5.

By studying the regulation and functions of HPV proteins, the virus life cycle will be better understood, expanding the opportunity to develop new diagnostic and therapeutic tools.

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Abbreviations

APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
BPV	bovine papillomavirus
CaMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
CDK	cyclin dependent kinase
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CKII	casein kinase II
CMV	cytomegalovirus
CRPV	cottontail rabbit papillomavirus
Da	Dalton
DAPI	4',6-diamino-2 -phenylindole
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTNB	5,5' –dithio-bis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
e.g.	exempli gratia (for example)
EGTA	ethylene glycol tetraacetic acid
EM	electrom microscopy
EV	epidermodysplasia verruciformis
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GSK-3	glycogen synthase kinase-3
GST	glutathione-S-transferase
h	hour

HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
His-E1^E4	C terminal hexa-histidine tagged 16E1^E4
HPV	human papillomavirus
HRP	horseradish peroxidase
i.e.	isoelectric focusing
IEF	id est (that is)
IgG	immunoglobin G
IPG	immobilised pH gradient
IPTG	isopropyl-beta-D-thiogalactopyranoside
IRES	internal ribosomal entry site
kb	kilo base
kDa	kilo Dalton
KLH	keyhole limpet haemocyanin
λ phosphatase	lambda phosphatase
LB	Luria Bertani
LCR	long control region
mcKLH	mariculture keyhole limpet haemocyanin
MALDI	matrix-assisted laser desorption/ionisation
MAPK	mitogen-activated protein kinase, also called ERK (extracellular signal-regulated protein kinase)
MBP	mannose binding protein
MEK	mitogen-activated or extracellular signal-regulated protein kinase kinase
mg	milligram
μg	microgram
min	minute
ml	millilitre
μl	microlitre
mm	millimetre
mM	millimolar
μM	micromolar
mRNA	messenger RNA
MS	mass spectrometry
m/z	mass/charge

MOI	multiplicity of infection
ng	nanogram
NLS	nuclear localisation signal
NP40	Nonidet P40
OA	okadaic acid
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pen/strep	cell culture antibiotic stock (penicillin/streptomycin)
pI	isoelectric point
PKA	protein kinase A (cAMP-dependent protein kinase)
PKB	protein kinase B, also called Akt
PKC	protein kinase C
pmoles	picomoles
PSD	post source decay
PVDF	polyvinylidene fluoride
rAd	recombinant adenovirus
rAdβGAL	βGAL-expressing recombinant adenovirus
rAdE1^E4	16E1^E4-expressing recombinant adenovirus
RNA	ribonucleic acid
R/T	room temperature
ROPV	rabbit oral papillomavirus
rpm	revolutions per minute
SB 3-10	3-(Decyldimethylammonio)propanesulfonate
SDS	sodium dodecyl sulfate
SV40	Simian virus 40
TOF	time-of-flight
TBE	tris borate EDTA
TBS	tris buffered saline
TEMED	N,N,N',N'- tetra-methyl-ethylenediamine
URR	upstream regulatory region
U-T	urea-thiorea

VEGF	vascular endothelial growth factor
VLP	virus-like particle
WT	wild –type

Chapter 1: Introduction

1.1 Viruses

Viruses are small (~ 10^{-8}-10^{-7} m) obligate parasites that replicate in host cells (Cann, 1997; Dimmock, 2001; Levine, 1996). They have a protein coat containing genetic material in the form of DNA or RNA, and sometimes an outer lipid envelope. Globally, viruses have been and will continue to be a major cause of disease, for example smallpox, influenza, polio, acquired immunodeficiency syndrome (AIDS) and Ebola. In the late 1800s, scientists working with plants infected with tobacco mosaic virus, first demonstrated transmission of an infectious agent that could pass through a porcelain filter known to block bacteria. The first filterable disease agent from animals, causing foot and mouth disease, was discovered thereafter. The disease agent was considered to be a 'contagious living liquid' for some time and it was not until 1939 that a virus, the tobacco mosaic virus, was first visualised by EM.

1.2 Introduction to papillomaviruses

Papillomaviruses are small (55 nm), non-enveloped, viruses with a double-stranded DNA genome of about 8 kb (Favre et al., 1997). They have an icosahedral protein coat (capsid) composed of the major viral capsid protein, L1 and the minor capsid protein, L2 (Zhou et al., 1991). Papillomaviruses infect epithelial tissues to cause proliferative lesions (or warts). More than 200 human papillomaviruses (HPVs) have been identified (de Villiers, 1999). Papillomavirus infections have also been detected in many higher vertebrates (Antonsson and Hansson, 2002), birds (Tachezy et al., 2002) and more recently, in aquatic mammals (Rector et al., 2004). Papillomaviruses are of clinical importance today because of their association with 99.7 % of cervical cancers (Walboomers et al., 1999). Worldwide, every year there are 450,000 new cases of cervical cancer and 300,000 deaths, 80 % of deaths being in less developed nations (Damasus-Awatai and Freeman-Wang, 2003).

1.3 Identification of papillomaviruses

Human warts were first shown to be infectious using cell-free filtrates in 1907 (Ciuffo, 1907). The first papillomavirus to be described was the cottontail rabbit papillomavirus (CRPV), originally called Shope papillomavirus after the discoverer (Shope and Hurst, 1933). The carcinogenic potential of a papillomavirus was first proven by inducing tumours in domestic rabbits using CRPV (Ito and Evans C.A., 1961). In 1959 bovine papillomavirus type 1 (BPV1) was shown to induce bladder tumours in cows (Olson et al., 1959). The first visualisation of papillomavirus particles in warts by EM was reported in 1949 (Strauss et al., 1949) and the first isolation of HPV DNA was in 1963 (Crawford and Crawford, 1963). The first HPV to be characterised was from a plantar wart (Klung and Finch, 1965; Rowson and Mahy, 1967). The existence of different HPVs was later demonstrated when plantar wart virus genes did not hybridise with genetic material from all warts tested (zur Hausen et al., 1974), so gradually more HPV isolates were discovered (Gissmann et al., 1977; Orth et al., 1977).

In the 1970's and 1980's interest grew in the role of HPVs in cervical cancer and they were finally recognised to be the causative agent of the second most common form of female carcinoma in the world (Boshart et al., 1984; Durst et al., 1983; zur Hausen et al., 1974).

1.4 Papillomavirus genomic organisation

Papillomaviruses have similar genomic organisation (Figure 1.1 shows HPV16 as an example of HPV genomic organisation) with well-conserved open reading frames (ORFs), all encoded on one DNA strand of the genome, with some degree of overlap. There is a non-coding region of DNA called the long control region (LCR) or the upstream regulatory region (URR; Figure 1.2). This contains the origin of DNA replication (ori; Chiang et al., 1992), an epithelial-specific enhancer element (containing AP-1 sites; Chong et al., 1991) and the early promoter (Cripe et al., 1987). Most papillomavirus types have eight ORFs; E1, E2, E1^E4, E5, E6, E7, L1 and L2, but some animal viruses have additional ORFs, for example, BPV1 has E3 and E8 (Stephens and Hentschel, 1987) and reindeer papillomavirus (RPV), European elk

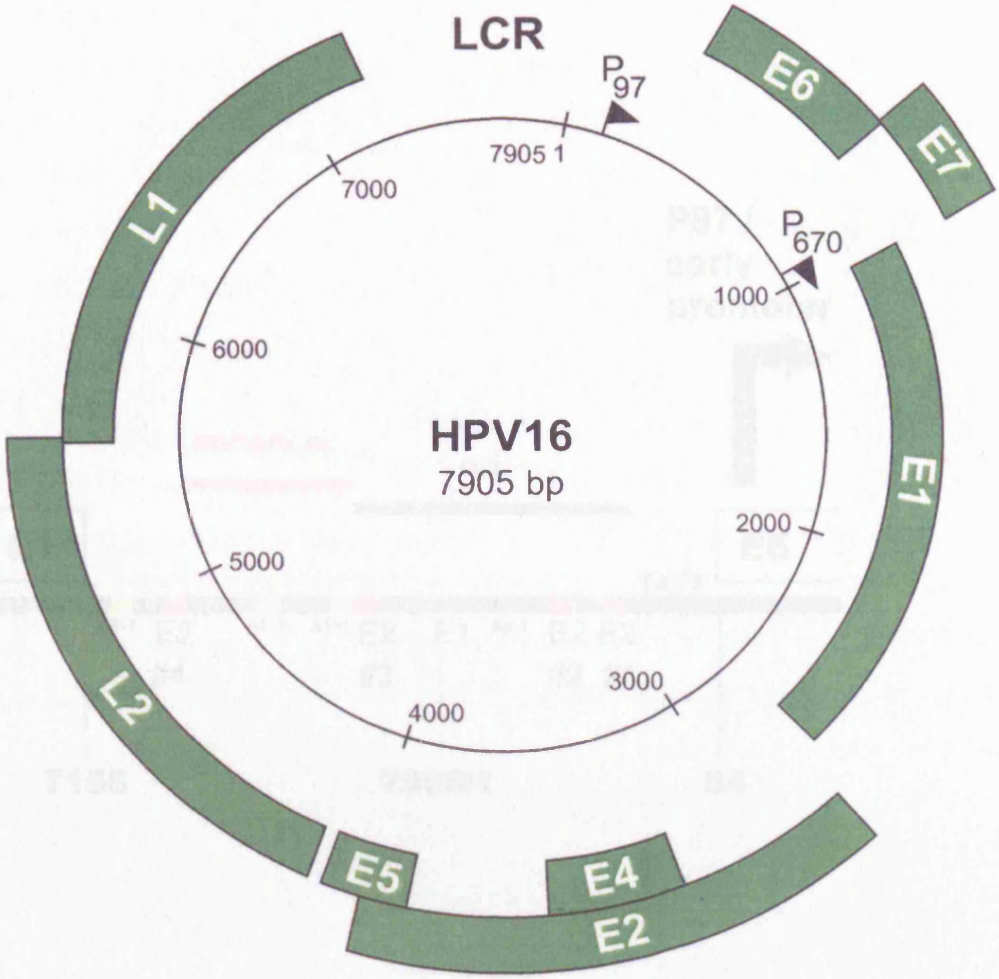


Figure 1.1 Genomic organisation of HPV16
The HPV16 genome is an 8 kb double-stranded DNA molecule that encodes eight ORFs. The two major promoters which regulate viral gene transcription are the early promoter, p97, and the late promoter, p670, which are named according to their nucleotide position. The LCR is a non-coding region but has regulatory sequences for replication and transcription.

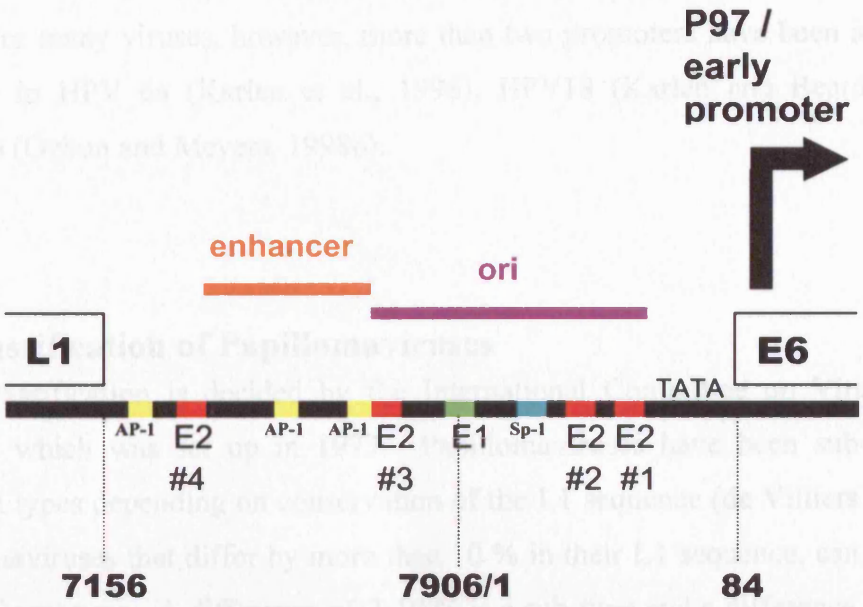


Figure 1.2 Schematic representation of the HPV16 LCR
The important elements of the LCR and the flanking ORFs are indicated in the diagram, and some nucleotide positions shown (not to scale). The relative positions of the origin of replication (ori), epithelial-specific enhancer, E1 and E2 binding sites (labelled #1-4) are shown. The binding sites of the transcription factors AP-1 and Sp1 are also indicated.

papillomavirus (EEPV) and deer papillomavirus (DPV) have E9 (Eriksson et al., 1994).

Genes are first transcribed from the early promoter and subsequently, the late promoter becomes active, however, the late promoter is dependent on the differentiation of the epithelium (Grassmann et al., 1996; Ruesch et al., 1998). The ORFs can be loosely described as 'early' or 'late' depending on which promoter they are mainly expressed from. For many viruses, however, more than two promoters have been identified, for example in HPV 6a (Karlen et al., 1996), HPV18 (Karlen and Beard, 1993) and HPV31b (Ozbun and Meyers, 1998b).

1.5 Classification of Papillomaviruses

Virus classification is decided by the International Committee on Viral Taxonomy (ICTV), which was set up in 1973. Papillomaviruses have been sub-divided into different types depending on conservation of the L1 sequence (de Villiers et al., 2004). Papillomaviruses that differ by more than 10 % in their L1 sequence, can be described as a different type. A difference of 2-10 % is a sub-type and a difference of < 2 % is a variant.

Papillomaviruses and polyomaviruses were first grouped together in the *Papovaviridae* family. However, the two virus groups were later recognised as being significantly different, so they were then classed as two separate families, *Papillomaviridae* and *Polyomaviridae.* In the late 1970's when new papillomavirus isolates were being found, the numbering system, e.g. 'HPV1', 'HPV11', became useful. In attempts to correlate the L1 sequence homologies with biological and pathological features of the virus, descriptions of phylogenetic groups, genera and species have emerged, the most recent system described by de Villiers et al. (2004; see Appendix.1). For example, the genus of alpha papillomaviruses includes all the species associated with genital lesions and the genus of beta papillomaviruses include species that frequently cause lesions in epidermodysplasia verruciformis (EV) patients. Furthermore, viruses can be classed as cutaneous or mucosal, depending on their preferred epithelial tissue tropism, or low-, intermediate- or high-risk, depending on their ability to cause cancer (Lorincz et al., 1992; Lowy and Howley, 2001).

1.6 Diseases associated with papillomaviruses

The majority of papillomavirus infections lead to proliferative, localised lesions in the infected stratified squamous epithelium (Figure 1.3). In humans, these epithelial sites are either in skin (cutaneous epithelium) or ano-genital areas, oral cavity or larynx (mucosal areas). The lesions may be referred to as warts and those in genital sites can be alternatively described as condylomas. Most of these lesions will spontaneously regress after months or years of persistence. However, in a small percentage of infections of 'high-risk' HPVs or certain animal papillomaviruses, the lesion can progress to malignancy (Lowy and Howley, 2001).

1.6.1 Site of infection; the epithelium

Papillomaviruses enter the stratified squamous epithelium by a microwound and infect the basal cells. The viral life cycle is tightly linked to the differentiation of the epithelium (Howley and Lowy, 2001).

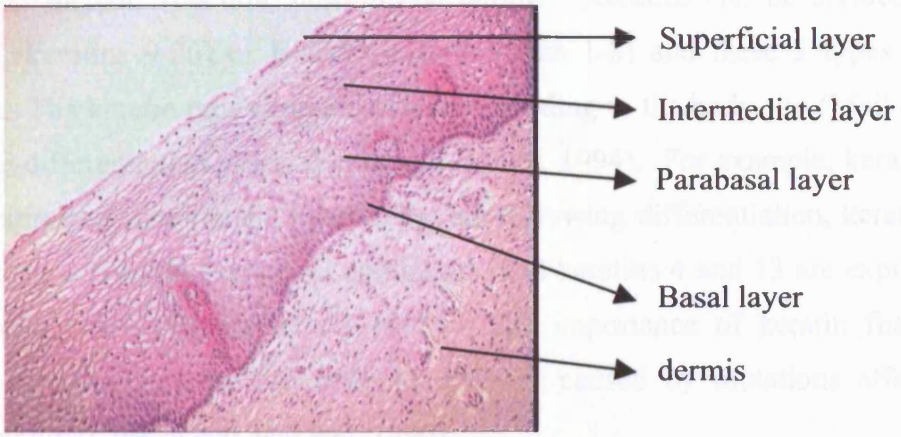
Epithelial tissue is avascular and lines internal cavities and external body surfaces. The terminology used to describe epithelium, is based on the structure and is defined below.

simple	one cell layer thick
stratified	two or more cell layers
squamous	cell width > cell height
cuboidal	cell height = cell depth
columnar	cell height > cell width

The predominant cell type is the keratinocyte, but antigen-presenting cells (Langerhans' cells) and sensory cells (Merkel's cells) are also present. In addition, cutaneous epithelium (skin) contains melanin-producing cells (melanocytes). The deepest layer of the epithelium is the basal layer, a single layer of mitotically active cells. The keratinocytes arise by division of the basal cells. Keratinocytes undergo a program of differentiation as they move upwards through the epithelial layers, and finally, they are sloughed off from the superficial or cornified layer (Fuchs, 1993; Ross M. H., 2003).

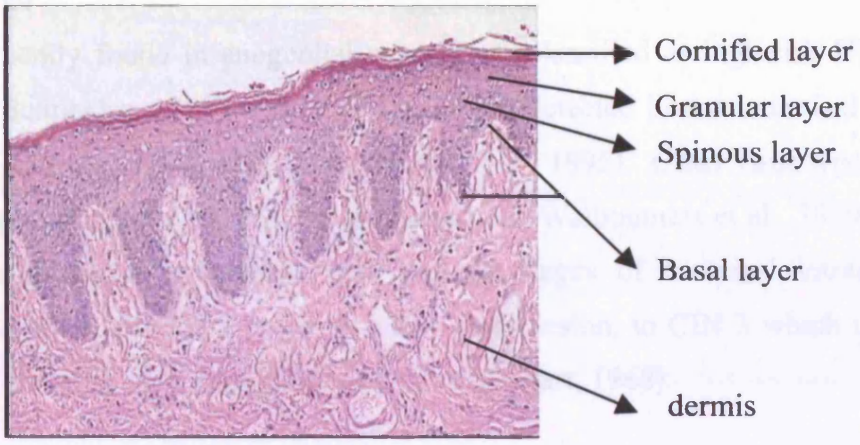
Figure 1.3 Stratified squamous epithelium
Haematoxylin and Eosin stains (H and E) of cervical epithelium and skin are shown (taken from http://www.inc58.pwp.blueyonder.co.uk/MicroAnatomy/epithelia/) as examples of mucosal and cutaneous epithelia, respectively. The characteristic layers of these types of epithelia are also indicated, and described in brief in the table.

Cervical Epithelium (mucosal)



Cutaneous	Mucosal	Charactersistics
Cornified	Superficial	filled with keratin filaments, cells are flattened, nuclei lost in cutaneous, cornified cell envelope in cutaneous (made of loricrin, involucrin, filaggrin & keratin)
Granular		contain keratohyalin granules (made of filaggrin precursors and loricrin)
	Intermediate	keratin filaments form
Spinous	Parabasal	post-mitotic, differentiation specific keratins, cytoplasmic extensions / spines in cutaneous,
Basal	Basal	mitotically active

Skin (cutaneous)



Keratins constitiute up to 85 % of the total protein in a fully differentiated keratinocyte (Ross M. H., 2003). They belong to the intermediate filament family of proteins and other members include vimentin, desmin and lamins. Keratins can be divided into acidic/type I (keratins 9-20) or basic/type II (keratins 1-8) and these 2 types form heterodimers. The keratin type expressed varies according to the body site (Moll et al., 1982) and the differentiation status (Fuchs and Weber, 1994). For example, keratins 5 and 14 are expressed in epithelial basal cells, but following differentiation, keratins 1 and 10 are expressed in the cutaneous epithelium, and keratins 4 and 13 are expressed in mucosal epithelium (Omary et al., 1998). The importance of keratin filament formation is demonstrated by the array of diseases caused by mutations affecting filament assembly (Corden and McLean, 1996).

1.6.2 Pathology of human papillomavirus infections

1.6.2.1 Benign lesions

Infection of low-risk HPVs are associated with benign lesions. These are mostly harmless and common within the population. For example, HPV1 can cause plantar warts and verrucas (Egawa et al., 1993) and HPV2 also causes common warts. Interestingly, HPV2 can occasionally also infect mucosal tissues such as genital or nasopharyngeal areas (Chan et al., 1997). Most cases of genital warts are caused by HPV6 and 11 (Chang et al., 1990), but these types can also occasionally cause laryngeal lesions (Quiney et al., 1989). Although benign, genital warts are particularly troublesome because of their unsightly appearance and tendency to reoccur after treatment, making them problematic for health authorities (O'Mahony, 2005).

1.6.2.2 Cancer

Viruses frequently found in anogenital cancers are classified as high-risk HPVs (zur Hausen and Schneider, 1987). HPV16 and 18 are detected in most cervical cancers, with type 16 causing ~50 % of all cases (Bosch et al., 1995). Other virus types seen in cervical neoplasia include 31, 33, 39, 45, 52 and 58 (Walboomers et al., 1999). HPV-induced transformation progresses with defined stages of 'cervical intraepithelial neoplasia' (CIN), from CIN 1 which is a low-grade lesion, to CIN 3 which is a high-grade lesion showing severe dysplasia or cancer (Richart, 1968).

Mucosal HPVs are also linked to cancers of the oral cavity and larynx (de Villiers et al., 1985; Scheurlen et al., 1986; Scully, 2005). Some cutaneous HPVs are associated with non-melanoma cancers of the skin and this was originally found in patients with the disease, EV. This is a rare inherited cutaneous disorder, possibly associated with truncating mutations of the *EVER1* or *EVER2* genes, the functions of which are unknown (Ramoz et al., 2002), or polymorphisms in the promoter of the *IL-10* gene that cause low expression of the cytokine (de Oliveira et al., 2003). The disease is characterised by persistent lesions, a high susceptibility to some HPVs, described as EV HPVs (e.g. HPV5, 8, 12 and 23), and about half of patients suffer from squamous cell carcinoma (Orth et al., 2001). A significant association has been found between non-melanoma skin cancer and EV HPV DNA but not other types of cutaneous HPV DNA (Harwood et al., 2004).

The prevalence of cervical cancers, especially in developing countries, makes HPV a worldwide burden, but considering its roles in vulvar, penile, anal, oral, laryngeal and non-melanoma skin cancers, HPV may account for almost 10 % of all cancers (Parkin et al., 2001; Zur Hausen, 1996).

1.7 Cervical cancer prevention

1.7.1 Education

A recent survey (September 2005) by Cancer Research UK, suggested that 2/3 of British women are not aware that smear tests are for cancer prevention. A similar study revealed that less than 1 % of British women surveyed were able to name HPV as a risk factor for cervical cancer (Waller et al., 2004). There is clearly a gap in knowledge, however, the need for education in countries with organised screening programmes is not as great as the need in less developed countries where the cervical cancer death incidence is 11.2 per 100,000 (compared to 3.1 per 100,000 in the U.K; *from* GLOBOCAN 2002, http://www-dep.iarc.fr/).

1.7.2 Screening and diagnosis

Pap smears have reduced the incidence of cervical cancer deaths by 70-75 % in many countries since their introduction around 50 years ago (Bergstrom et al., 1999). This method of detecting cytological abnormalities in smears of cervical cells, was

developed by George Papanicolaou (Papanicolaou, 1941). Although, this strategy has proved to be a great success story of cancer screening, smear tests are laborious to analyse and give a high rate of false-negatives (Mannino, 1998). In the near future, several countries are likely to convert to liquid-based cytologic collection instead of smears on slides, and this, together with computer-automated rather than manual analysis, should provide more sensitive and more cost-effective screening (Austin and Ramzy, 1998). Another basis for screening is to actually test for the presence of HPV DNA (Lorincz, 1996), and although accuracy for virus presence is high, the results may cause undue stress in patients as the majority of high-risk HPV infections will clear naturally and not progress to cancer. A more useful approach could be to use cellular markers for cervical dysplasia, for example, increased staining of mini chromosome maintanance proteins (MCMs) and CDC6 (Williams et al., 1998), and $p16^{INK4A}$ (Klaes et al., 2002). MCMs and CDC6 are required for human DNA replication and $p16^{INK4A}$ is a cellular inhibitor of CDK4/6, and these markers may be upregulated in CINs as a consequence of E7 activity.

1.7.3 Prophylactic vaccine

As for any other infectious disease, the vaccination approach for cervical cancer prevention has been attractive for some time. Early studies suggested that the E7 (Tindle et al., 1991) and L1 (Jenkins et al., 1990) proteins have immunogenic potential. L1 was then shown to induce specific neutralising antibodies in rabbits when allowed to self-assemble into virus-like particles (VLPs) but not when injected as monomers (Kirnbauer et al., 1992). VLP vaccine research therefore dominated (Chen et al., 2000; Evans et al., 2001) and then in 2002, scientists announced 100 % efficacy in preliminary trials of a HPV16 L1 VLP vaccine (Koutsky et al., 2002). Immunology studies suggest that this type of vaccine will induce sufficient neutralising antibody production and cell-mediated immune response (T cell proliferation and cytokine production) for a prophylactic effect (Harro et al., 2001; Pinto et al., 2003). Merck and GlaxoSmithKline are currently developing VLP vaccines. Merck recently (October 2005) announced the success of its VLP vaccine in phase III trials, and hope that this vaccine, which targets HPV types 16, 18, 6 and 11 (named GARDASIL™), will be marketed in 2006.

The vaccine success is certainly a great advancement in cervical cancer and genital wart therapy, however, there are limitations to its potential. L1 is not expressed in HPV-transformed cells or in the lower epithelial layers where there is greater immune surveillance, so the vaccine will be effective against new infection by viruses, but not against existing infections or cancer. For this reason, the vaccine is likely to be administered only to girls before they become sexually active. Therefore, the need for a therapeutic vaccine or continued cervical screening still remains for the present generation. An additional problem is that the current vaccines will not give 100 % protection against cervical cancer or genital warts because types 16 and 18 cause ~70 % of all cervical cancers and types 6 and 11 cause ~90 % of all genital warts (Shaw, 2005). Development of vaccines that cover a broader range of HPVs may therefore prove to be necessary.

Research continues for a therapeutic vaccine that will provoke a sufficient cell-mediated immune response for clearance of virus-infected or virus-transformed cells. The majority of studies use E6- or E7-targeted approaches (Baldwin et al., 2003; Hallez et al., 2004), but E2 has also been investigated for immunogenicity (Brandsma et al., 2004; Corona Gutierrez et al., 2004).

1.8 Progression to cancer

Upregulation of the viral oncogenes E6 and E7 is required to initiate and maintain transformation, and this is attributed to their ability to disregulate normal cellular controls for example by inactivating p53 and pRB (retinoblastoma protein) respectively (Kubbutat and Vousden, 1996; See 1.13). E6 and E7 levels are generally low in benign lesions, but in cancer, there is high expression throughout the epithelium (Durst et al., 1992). Cellular disregulation caused by the HPV oncogenes leads to genomic instability (Duensing and Munger, 2004) which contributes to the development of the hallmarks of cancer (Hanahan and Weinberg, 2000). E5 may also have a role in initiating transformation although it is not required to maintain transformation (Leechanachai et al., 1992).

An important step that accelerates cancer progression is the integration of the viral DNA into the host DNA. In benign infections or low grade CINs, HPV genomes are

maintained as autonomously replicating episomes, whereas in high grade CINs and cancer, the viral DNA is frequently integrated into the host cellular genome (Durst et al., 1985; Melsheimer et al., 2004). Integration does not appear to occur at specific sites in the host DNA, although common fragile sites (CFSs) may be preferred. Examples of such sites favoured by HPV16 include regions 13q22, 3p14 and 17q23 (Thorland et al., 2003). Integration can cause alterations in host gene expression, for example, disruption of chromosome segment 3p14, causes the downregulation of a candidate tumour suppressor gene, fragile histidine triad (*FHIT*; Hendricks et al., 1997). In some cases integration has occurred near cellular oncogenes such as *c-myc*, particularly with HPV18 (Durst et al., 1987; Ferber et al., 2003). Integration does seem to follow a pattern in context of the viral DNA; integration in the E1 or E2/E4 ORF appears to be favoured for the progression of cancer (Baker et al., 1987; Schwarz et al., 1985). E2 is able to repress expression of E6 and E7 and both E2 and E1^E4 can induce growth arrest and apoptosis (see 1.13). These integration events therefore cause the upregulation of E6 and E7, and give the cells a growth advantage that may lead to cancer.

Integration and the activity of E6 and E7 can also give rise to structural changes in the host genome. One mechanism is abnormal centrosome duplication, which can cause the loss or gain of chromosomes (Duensing et al., 2000). A common example of genetic instability in cervical cancers, is the presence of multiple copies of the long arm of chromosome 3 (Heselmeyer-Haddad et al., 2003). This region encodes the RNA component of the telomerase gene, *TERC*, so its overexpression aids transformation.

1.9 Transmission of human papillomaviruses

Papillomaviruses do not have lipid envelopes, so are relatively resistant to dessication and have been shown to remain infectious after dehydration and heating to 65 °C (Roden et al., 1997). These viruses are therefore easy to transmit, for example, barefoot walking in swimming pools is associated with the spread of verrucas/plantar warts (Vaile et al., 2003). Anogenital infections are mainly acquired by sexual contact (Kjaer et al., 2001) and the presence of anogenital HPV types in oral lesions, indicates transmission by the genital-oral route (Scully, 2005). However, the rare detection of anogenital HPVs in virgins and in children, suggests that there are other modes of

transmission, for example from mother to child during childbirth (Cason and Mant, 2005) or from environmental surfaces (Strauss et al., 2002).

1.10 Papillomavirus receptor and entry

Papillomaviruses must enter the basal cells of the epithelium in order to establish an infection and this access is likely via an epithelial microwound (Doorbar, 2005). It has been suggested that for infections to persist, viruses must also infect epithelial stem cells (Egawa, 2003). The major capsid protein, L1 is considered to be more important than the L2 protein for viral attachment and entry (Volpers et al., 1995).

Studies on HPV6 proposed that alpha-6-integrin is the cellular receptor for entry (Evander et al., 1997), however, further experiments showed that alpha-6-integrin was not required for either BPV4 virions or HPV11 VLPs (Joyce et al., 1999; Sibbet et al., 2000). Instead, several studies suggested that heparin sulphate is necessary for viral entry (Combita et al., 2001; Giroglou et al., 2001; Joyce et al., 1999). Indeed, heparin sulphate has been shown to be the receptor for several viruses including herpesvirus (Lycke et al., 1991). However, the earlier studies claiming the importance of heparin suphate, had used VLPs rather than actual infectious viruses. When HPV31 infectious viruses were investigated, it was found that heparin sulphate was involved in infection of COS-7 cells (transformed monkey kidney cell line) and C33A cells (HPV-negative cervical cancer cell line) but not required for infection of HaCaT cells (HPV-negative human keratinocyte cell line that can undergo differentiation; Patterson et al., 2005). This suggests that more investigation is needed, particularly using real viruses and the natural target cells.

Little is known about the steps involved in viral entry and subsequent events, however, studies suggest that HPV VLP entry and uptake is slow compared to other viruses, and dependent on clathrin-mediated uptake (Day et al., 2003) and the endosomal pathway (Selinka et al., 2002).

1.11 The study of papillomavirus infections in the laboratory

Investigation of real infections is a good approach to study the papillomavirus life cycle, so animal papillomavirus infections of the natural hosts and analysis of human clinical samples, have been widely used by scientists. However, using animal models is controversial and not always ideal for representing infections in humans, and clinical material is difficult to obtain and biopsies mostly demonstrate dysplasia, rather than a productive viral infection. For more functional studies and manipulative experiments, growing viruses in cells is desirable. However, papillomaviruses do not propagate in normal monolayer cell culture (Lowy and Howley, 2001). For this reason, other strategies have been employed and these are described below.

1.11.1 Xenotransplantation

Human epithelia can be excised then experimentally infected with virus particles, and to complete the viral life cycle, this tissue can be implanted into research animals. Examples of this xenotransplantation technique include the implanation of HPV-infected human foreskin tissue under the kidney capsule of athymic mice (Kreider et al., 1986) and the grafting of human foreskin cells onto the back of Severe Combined Immunodeficiency (SCID) mice followed by inoculation of the human cells with HPV (Bonnez, 1998; Brandsma et al., 1995).

1.11.2 Cell culture

Papillomavirus studies using undifferentiated keratinocyte monolayer cultures have been satisfactory for studying the early stages of the virus life cycle, but viral genome amplification and late viral protein expression is dependent upon keratinocyte differentiation (Bedell et al., 1991). However, there are methods to induce differentiation of monolayer keratinocytes, for example by using medium with a high calcium concentration (Stanley and Yuspa, 1983) or by suspending in a semisolid medium such as methylcellulose (Ruesch et al., 1998).

1.11.3 Epithelial raft culture

HPV-infected epithelia can now be grown from HPV-containing primary basal keratinocytes or keratinocyte cell lines, using the raft system (Blanton et al., 1991;

Dollard et al., 1992; Flores et al., 1999; Meyers et al., 1992). Epithelial rafts are made by growing keratinocytes at the air-medium interface, on a collagen matrix that contains fibroblasts. Nutrients and necessary growth and differentiation factors are present in the medium and reach the keratinocytes by capillary action through the collagen. In this way, a culture that resembles differentiated epithelium can be produced and HPV infections can be mimicked.

The raft system provides an excellent tool for functional studies, as mutant HPV genomes can be expressed and analysed. Examples include knockouts of HPV16 E5 and HPV31 E1^E4 genes (Genther et al., 2003; Wilson et al., 2005).

1.12 An outline of the papillomavirus life cycle

Following viral entry in basal keratinocytes, the viral genome is replicated and maintained at a low copy number. Approximately 10-200 copies of the viral episomal DNA are maintained at this stage, and the early viral proteins, E6, E7, E1 and E2, are expressed at low level (De Geest et al., 1993; Stanley et al., 1989). This genome maintenance phase is a necessary early infection step, but is prolonged during latent or subclinical papillomavirus infections. In latent infections, E1 is perhaps the main viral protein expressed as it is necessary for viral genome replication (Zhang et al., 1999).

During the next phase of the viral life cycle, cell proliferation is stimulated so that viral genome amplification can occur. This is achieved by E6 and E7, overriding normal cell cycle regulation by targeting the cellular proteins p53 and pRB respectively. Genome amplification favours a cycling host cell because this can provide all the necessary cellular factors, such as replication protein A (RPA) and DNA polymerase α (Wilson et al., 2002). Before viral genome amplification can occur, levels of E1, E2, E1^E4 and E5 increase due to activation of the late (or differentiation dependent) promoter (Hummel et al., 1992; Middleton, 2003). E1 is essential for genome amplification as it acts as a DNA helicase to unwind the viral genome, and E2 is needed to recruit E1 to the viral DNA, so an increase in both proteins correlates with an increase in genome amplification (Ozbun and Meyers, 1998a). E1^E4 (Peh et al., 2004) and E5 (Fehrmann et al., 2003) are also believed to be necessary for efficient genome amplification and other late viral events.

Finally, for completion of the life cycle, new capsid proteins must be expressed to package the newly amplified genomes. L1 and L2 expression begins in a subset of E1^E4-expressing cells in the upper layers of the epithelium (Doorbar et al., 1997). L2 translocates to the nucleus and then recruits L1 to the nucleus and it is here that encapsidation of the viral genome is thought to occur (Becker et al., 2004; Florin et al., 2002a). The capsid is assembled in an icosahedral arrangement, consisting of 360 copies of L1 arranged as 72 pentamers, and about 12 copies of L2 (Finnen et al., 2003). Virus transmission is achieved by shedding of cells of the upper epithelial layer (Bryan and Brown, 2001). The virus E1^E4 protein may facilitate transmission by making keratinocytes more fragile by pertubation of normal cytokeratin networks (Doorbar et al., 1991) and by affecting cornified envelope assembly in the skin (Brown and Bryan, 2000).

The functions and characteristics of each HPV protein is further detailed in section 1.13.

1.13 The human papillomavirus proteins

1.13.1 The E6 protein

The E6 protein is a small, basic protein, with two zinc finger domains (Barbosa et al., 1989). E6 of high-risk viruses also have a PDZ binding motif (Kiyono et al., 1997). The full-length protein is the predominant form, but different truncated splice variants, called E6*(I-IV), have also been detected (Smotkin et al., 1989). E6 has a major role in cellular transformation (Bedell et al., 1987; Munger et al., 1989a).

1.13.1.1 Association with p53

The ability of E6 to contribute to transformation is largely due its ability to inactivate the cellular tumour suppressor, p53. E6 of low-risk and high-risk viruses can bind p53 (Crook et al., 1991; Werness et al., 1990). However, only E6 from high-risk viruses can promote the degradation of p53 (Crook et al., 1991; Scheffner et al., 1990). Degradation is achieved by E6 recruiting a cellular ubiquitin ligase, E6-associated protein (E6-AP), so that a complex of E6/p53/E6-AP forms, resulting in the ubiquitination and degradation of p53 via the ubiquitin-proteasome pathway (Scheffner

et al., 1993). p53 has a pivotal role in the regulation of many cellular events including apoptosis, DNA repair, cell cycle checkpoints and senescence (Levine, 1997; Zhao et al., 2000). p53 inactivation, and indeed several other anti-apoptotic effects of E6, are thought to have evolved as a safety mechanism to avoid the cell's natural responses to viral replication and E7-driven proliferation. For example, it has been shown that for maintenance of high-risk viral episomes, p53 must be inactivated, since p53 may have a role in guarding the cell against maintaining extra-chromosomal DNA (Park and Androphy, 2002).

The truncated E6 proteins (E6*) may have a modulatory role, as demonstrated by their ability to block full-length E6-driven degradation of p53 *in vitro* (Pim et al., 1997). This type of modulation may be necessary as there is a fine balance between creating conditions perfect for viral growth and preventing transformation, which would result in abrogation of the virus life cycle.

Viruses that cannot degrade p53 but where the E6 still associates with p53, can also affect p53 activity, for example, by inhibiting p53 binding to DNA, thus affecting its normal transcription factor activites (Lechner and Laimins, 1994; Thomas et al., 1995).

1.13.1.2 Other cellular binding partners

Following the discovery that E6 binds p53 and E6-AP, several other cellular binding partners have been identified. The first was called E6-binding protein (E6BP), a homologue of ERC55, a calcium-binding protein (Chen et al., 1995a). The significance of the E6:E6BP interaction is unknown, although E6 has been shown to inhibit calcium-dependent differentiation of keratinocytes (Sherman and Schlegel, 1996).

Another protein shown to interact with BPV1 E6 and HPV16 E6 is paxillin (Tong and Howley, 1997). Paxillin transduces signals from the plasma membrane to focal adhesions and the actin cytoskeleton, and BPV1 E6 was found to disrupt actin filaments, the effects of which could be important for pathogenesis or malignancy.

E6 proteins are also capable of binding many PDZ domain-containing proteins (PDZ stands for the three proteins in which the domain was first identified; PSD-95, Dlg and ZO-1). The first interaction of this type to be recognised was the binding of high-risk E6 to hDlg (Kiyono et al., 1997) and the subsequent degradation of hDlg (Gardiol et

al., 1999). Other PDZ-containing proteins found to bind E6 and be targeted for degradation by the ubiquitin-proteasome pathway, include, MAGI-1, (Glaunsinger et al., 2000) MUPP1 (Lee et al., 2000b) and hScrib (Nakagawa and Huibregtse, 2000). PDZ domains are considered to act as a 'glue' in protein complexes, particularly in signal transduction complexes, cell junctions and plasma membrane-cytoskeleton interfaces and have roles in cell-cell contact and epithelial cell polarity (Fanning and Anderson, 1999). Therefore, E6 targeting of these proteins may disrupt normal cellular controls, possibly contributing to a transformed phenotype.

Other proteins that E6 can bind and promote the degradation of, are c-myc (Gross-Mesilaty et al., 1998), bak (Thomas and Banks, 1998) and MCM7 (Kuhne and Banks, 1998). c-myc is a transcription factor that can have pro-apoptotic effects (Pelengaris and Khan, 2003), bak is a pro-apoptotic member of the Bcl-2 family (Korsmeyer et al., 2000) and MCM7 has a role in licensing DNA replication once per cell cycle (Blow and Hodgson, 2002), so targeting these proteins would be advantageous for the survival and successful replication of HPV. E6 has also been shown to bind but not degrade p300/CBP, a p53 co-activator (Patel et al., 1999), and by doing so, may reduce p53/p300/CBP-dependent transcription. Transactivation by HPV E2 also appears to involve co-activation by p300/CBP (Lee et al., 2000a), so E6 may also inhibit E2-driven transactivation.

1.13.1.3 Effects on transcription of cellular proteins

E6 can activate telomerase (Klingelhutz et al., 1996), a ribonucleoprotein enzyme important for the maintenance of telomeric structures at the ends of chromosomes (Greider, 1996). Lack of telomeric activity leads to progressive erosion of the ends of chromosomes, leading to senescence, a normal cellular event to limit the cell's life span and prevent genomic instability and mutations. The catalytic subunit of telomerase, hTERT, is upregulated in cells expressing E6. Possible mechanisms have been proposed, for example, E6/myc complexes targeting myc-resposive elements in the *hTERT* promoter (Veldman et al., 2003) and E6-AP-mediated transactivation, with E6 recruiting E6-AP to the promoter (Liu et al., 2005).

E6 can also upregulate the transcription of vascular endothelial growth factor (*VEGF*), and although the mechanism is unknown, it is dependent on the Sp-1 transcription factor binding sites in the *VEGF* promoter (Lopez-Ocejo et al., 2000).

1.13.1.4 Effects on viral transcription

The presence of an E6-inducible enhancer in the URR was reported (along with two other enhancers) for HPV18 (Gius et al., 1988). A subsequent report, however, described that full-length E6 repressed the HPV16 early promoter, while the truncated E6, E6*I, transactivated this promoter (Shirasawa et al., 1994). It is also feasible that by E6 interacting with some cellular factors, viral transcription is affected, for example p300/CBP can transactivate the viral early promoter but E6 can inhibit this factor by binding it (Patel et al., 1999).

1.13.1.5 DNA binding

The DNA-binding motif, the zinc finger domain, has been proposed to be present in HPV E6 for some time (Barbosa et al., 1989), however it was not until recently that the DNA-binding properties of E6 have been fully investigated (Ristriani et al., 2000). The C-terminal zinc finger has been shown to recognise 4-way DNA junctions, in a DNA-structure specific manner (Ristriani et al., 2001). The significance is unclear, although, the recognition of DNA structures may be related to the E6-inducible enhancer described above.

1.13.1.6 Immune evasion

It has been shown that HPV16 E6 can down-regulate cell surface E-cadherins, leading to a loss of Langerhan's cells in the epithelium (Matthews et al., 2003). Langerhan's cells are antigen presenting cells and are crucial in the immune response to HPV infection, so their loss contributes to immune evasion by HPV (Bedell et al., 1987; Tindle, 2002).

1.13.1.7 Association with E2

Recently, Grm et al. (2005) showed that E6 of HPV16 and HPV18 can bind to 16E2. E6 and E2 colocalise in cells preferentially forming discrete nuclear speckles, and it appears that E2 can stabilise E6 protein levels especially that of E6*I. Also, E2 inhibits the ability of E6 to cause degradation of the PDZ-containing proteins, MAGI-1 and MAGI-3, but does not affect the degradation of p53. This interaction also modulates the ability of E2 to affect viral replication and viral transcription (see 1.13.4.7).

1.13.2 The E7 protein

E7 is a small acidic protein, with a zinc finger domain and an LXCXE motif, required for binding to pRB (Stacey et al., 1994; Vousden, 1993). It has sequence and functional similarities to other DNA tumour virus proteins, such as SV40 large tumor antigen (TAg) and adenovirus E1A (Barbosa et al., 1990). E7 has a major role in cellular transformation (Bedell et al., 1987; Munger et al., 1989a).

1.13.2.1 Association with retinoblastoma family proteins

The abilty of E7 to bind to members of the retinoblastoma protein family (pRB, p107, p130) is the most characterised property of this viral protein (Davies et al., 1993; Dyson et al., 1989; Munger et al., 1989b). In a similar way to adenovirus E1A (Ludlow et al., 1989) and SV40 TAg (Dyson et al., 1992), E7 was found to bind preferentially to hypophosphorylated retinoblastoma proteins.

The function of hypophosphorylated pRB is to bind transcription factors of the E2F family, preventing E2F activating S-phase specific genes. As cells go through the cell cycle from G0 to G1 and S-phase, pRB gets progressively phosphorylated by cyclin-dependent kinases (CDKs), enabling E2F to be released and activate S-phase genes such as those involved in DNA synthesis (Dyson, 1998). By binding pRB, E7 can disrupt pRB-E2F complexes, leaving E2F free to activate genes that cause cell cycling. E7 from both low- and high-risk HPVs can bind pRB but the binding affinity appears to be greater with high-risk types (Gage et al., 1990).

1.13.2.2 Effects on other cell cycle regulators

As a consequence of E2F activation, several cell cycle regulatory proteins are also affected by E7. One example is the upregulation of CDK2/cyclin E (Funk et al., 1997; Jones et al., 1997), a result of which is increased DNA synthesis and further inactivation of pRB by phosphorylation. CDK2 can be inhibited by the CDK inhibitors, $p21^{CIP}$ and $p27^{KIP}$, and reports of 16E7 binding these inhibitors, suggest another mechanism by which CDK2/cyclin E is activated by 16E7 (Funk et al., 1997; Jones et al., 1997). Once again, E7 is comparable to adenovirus E1A, since E1A has been shown to inhibit $p27^{KIP}$ (Mal et al., 1996). 16E7 has also been shown to directly interact with CDK2/cyclin A *in vitro* and increase the kinase activity of the complex (He et al., 2003). CDK2/cyclin E but not CDK1/cyclin B kinase activity was also increased by addition of 16E7. This supported the finding in a previous study where

CDK2 and cyclin A coimmunoprecipitated with E7 extracted from HPV16-transformed cervical cells (Tommasino et al., 1993).

1.13.2.3 Other cellular binding partners

E7 can associate with histone deacetylase (e.g. HDAC-1) complexes indirectly through the Mi2β protein (Brehm et al., 1999). Histone deacetylation is associated with chromatin taking on a 'closed' conformation, thus resulting in transcription repression (Pazin and Kadonaga, 1997). pRB can associate with HDAC-1 and both proteins cooperate to repress E2F-regulated promoters (Brehm et al., 1998), so E7 may interfere with formation of the pRB/HDAC-1 complex.

E7 can also bind members of the AP-1 transcription factor family, such as c-jun and c-fos, enhancing their transactivating properties (Antinore et al., 1996). AP-1 transcription factors promote cell proliferation and regulate differentiation (Weiss and Bohmann, 2004), so this activity of E7 causes cell cycle progression. Conversly, E7 can also inhibit c-jun activity. The *c-jun* promoter itself, contains AP-1 binding sites, and can normally be regulated by c-jun complexes. One such complex has been shown to be pRB/c-jun (Nead et al., 1998) and E7 can disrupt this complex and therefore affect c-jun activity in a negative manner. This positive or negative effect of E7 on c-jun may reflect the need to promote cell progression at one time and deregulate keratinocyte differentiation at another time (Chakrabarti and Krishna, 2003).

Other targets are members of the cellular basal transcription machinery, TATA binding protein (TBP; Massimi et al., 1996) and TBP-associated factor-110 (TAF-110) (Mazzarelli et al., 1995). The significance is unknown, but hints at E7's ability to cause global effects on transcription.

Similarly to HPV E6 and adenovirus E1A (Shuen et al., 2003), HPV E7 can also bind p300/CBP leading to repression of the co-transactivating role of p300/CBP (Bernat et al., 2003). As discussed for E6, this event may reduce transactivation by HPV E2.

A novel binding partner of E7 is the cellular phosphatase, PP2A (Pim et al., 2005). E7 expression was found to upregulate PKB/Akt signalling, which triggers a pro-survival pathway. PP2A can inactivate PKB by dephosphorylation, but E7 binding is able to inhibit this, thus prolonging the PKB signal.

1.13.2.4 Association with E2

Recently, Gammoh et al. (2006) observed that HPV16 E2 could inhibit the transforming activity of E7 (when expressed with an activated ras oncogene) without lowering E7 transcription. Further investigation showed that E7 and E2 can interact *in vitro* and in cells. The region of E7 involved in E2 binding is the same as that occupied by some cellular binding partners, such as and Mi2β and TBP. E2 may interfere with E7 binding of these cellular proteins, so this could be the mechanism of the reduced transforming activity. In addition, E2 can increase the half-life of E7 and cause the relocation of E7 to mitotic chromosomes, an event that does not occur when E7 is expressed alone. This association with chromosomes could be important in the ability of E7 to cause genomic instability (Duensing and Munger, 2004).

1.13.3 The E1 protein

The E1 protein is the largest papillomavirus protein and is well-conserved. E1 proteins are generally acidic and they have a nuclear localisation signal (NLS), DNA binding domain and ATP-dependent helicase activity (Wilson et al., 2002).

1.13.3.1 Necessary for papillomavirus replication

After sequencing of HPV1a and BPV1 E1 ORFs, a role for papillomavirus E1 in replication was predicted, given its homology with the SV40 large T antigen, already known for replication initiation (Clertant and Seif, 1984). Consistent with this, it was shown that the E1 ORF is necessary for maintenance of the viral genome as an episome (Lusky and Botchan, 1985; Sarver et al., 1984). Subsequently, both HPV E1 and E2 were found to be necessary and sufficient for the replication of plasmids containing the ori (Kuo et al., 1994; Yang et al., 1991). E1 can also drive replication independently of E2, but only when expressed at high levels (Bonne-Andrea et al., 1995; Seo et al., 1993).

1.13.3.2 Helicase activity

Conserved motifs, typical of nucleoside triphosphate-binding proteins were identified in the C-terminal half of E1 (Gorbalenya et al., 1990). E1 of BPV1 was found to bind ATP (Sun et al., 1990) and similarly, HPV16 E1 was shown to have ATP-binding and ATPase activity (Raj and Stanley, 1995). Helicase activity was subsequently shown for

BPV E1 (Yang et al., 1993) and HPV E1 (Hughes and Romanos, 1993); E1 was found to be a 3' to 5' helicase that can unwind linear dsDNA fragments, circular dsDNA and ssDNA with a partially double-stranded region.

The ATPase and helicase activities are associated with the hexameric form of the protein (Fouts et al., 1999; Sedman and Stenlund, 1998). The formation of the final hexameric complex is stepwise and dependent on E2- and ori-binding (Chen and Stenlund, 2001; Sanders and Stenlund, 2000).

1.13.3.3 E1 assembly on the origin of replication

E2 binding of E1 is how E1 is targeted to the ori (Blitz and Laimins, 1991; Mohr et al., 1990). Although E1 can initiate replication independently of E2 when expressed at high levels, this is believed to occur through non-specific DNA binding (Dixon et al., 2000). *In vivo*, E1 is thought to be expressed at low levels (Seedorf et al., 1987), so E2 is needed to improve the efficiency of ori binding.

The DNA binding domain of E1 is in its N-terminal region and has been structurally analysed (Chen and Stenlund, 1998; Enemark et al., 2000). The region of E1 important for interacting with the transactivating domain (TAD) of E2, lies in the C-terminus/ATP-binding area (Titolo et al., 1999).

The E1 binding sites in the ori (E1BSs) were identified when BPV1 E1 was shown to bind preferentially to a hexanucleotide sequence, 5'-ATTGTT-3', in an E2-dependent manner (Chen and Stenlund, 2001). The ori of papillomaviruses have two copies of this E1BS and 4-5 copies of related hexanucleotide sequences, overlapping with each other in a 24 bp region of the ori. A stepwise mechanism for E1 loading onto ori has been proposed by Chen and Stenlund (2001). A complex involving two E1 molecules, two E2 molecules and ori first forms on a pair of E1BSs. Another complex of $E1_2$-$E2_2$-ori forms using another pair of E1BSs, but on the opposite face of the DNA helix (Chen and Stenlund, 2002). As a result, DNA at the ori is distorted and bent, ready for unwinding by the helicase (Gillette et al., 1994). The helicase activity does not initiate until E2 is displaced by ATP binding to E1 (Sanders and Stenlund, 1998) and E1 has oligomerised via its C-terminal to form two E1 hexamers (Chen and Stenlund, 2002; Sanders and Stenlund, 2000).

1.13.3.4 Cellular binding partners

Several cellular factors are involved in the replication of papillomaviruses (Melendy et al., 1995), and since SV40 TAg has been shown to bind several host proteins of the replication complex (Melendy et al., 1995), the ability of HPV E1 to bind replication-related proteins was also investigated.

The E1 of BPV1 was found to bind the DNA polymerase α primase subunit (Park et al., 1994), then the E1 of HPV16 was also shown to have the same binding activity (Masterson et al., 1998). HPV11 E1 was found to interact with histone H1 and was able to displace it, suggesting a need to disrupt nucleosomes and make the viral chromatin more accessible for replication (Swindle and Engler, 1998). Similarly, HPV18 E1 associates with Inil/hSNF5 (Lee et al., 1999), a component of the SWI/SNF protein complex, which has chromatin remodelling abilities and usually serves to facilitate transcription (Peterson and Herskowitz, 1992). In this case, Inil/hSNF5 binding appeared to be important for HPV DNA replication. Another E1 binding partner believed to be important in viral replication, is replication protein A (RPA), a ssDNA binding protein required in DNA replication (Iftode et al., 1999). HPV11 E1 was shown to bind the largest subunit of RPA, thereby recruiting RPA to the site of viral DNA replication (Loo and Melendy, 2004).

In addition to binding replication proteins, E1 of HPV11 can associate with the chaperone proteins, Hsp40 and Hsp70 (Liu et al., 1998). Chaperone proteins assist in the folding of proteins and the assembly of protein complexes (Hartl, 1996), and by EM, it was seen that the formation of E1 di-hexamers was facilitated by the interaction with Hsp40.

1.13.4 The E2 protein

The E2 protein possesses a transactivation domain (TAD) in its N-terminal region and a dimerisation and DNA-binding domains in its C-terminal (Giri and Yaniv, 1988). The N- and C-terminal domains are separated by a flexible hinge region and the protein exists as a dimer (Dostatni et al., 1988; Gauthier et al., 1991). mRNA encoding truncated E2 forms containing the C-terminal region (E2C), have also been detected (Doorbar et al., 1990; Lambert et al., 1987) and have proposed functions (Bouvard et

al., 1994b; Liu et al., 1995).

1.13.4.1 DNA binding

E2 binds DNA via its C-terminal to the palindromic consensus site, ACCgNNNNcGGT (where bases in small case are preferred and N_4 is the spacer between the two palindromic halves; Androphy et al., 1987; Hawley-Nelson et al., 1988). The spacer varies in its sequence but not in length, and different E2 proteins have different preferences for the sequence, for example, E2 of HPV16 has greater affinity for the spacer sequence, AA(A/T)N (Hines et al., 1998). High-risk HPVs have four E2 binding sites, E2BS#1-4, in their LCR (Figure 1.2). E2BS#1 is the most proximal to the early promoter and E2BS#4 is the most distal. E2 binds E2BS#4 with greatest affinity and E2BS#1 with lowest affinity, so E2BS#1 may only be occupied when E2 levels are high (Sanders and Maitland, 1994).

1.13.4.2 Transcriptional activation

A major function of E2 is to transactivate papillomavirus genes from the early promoter and this activity has been assigned to its N-terminal TAD (Cripe et al., 1987; Hirochika et al., 1987; Phelps and Howley, 1987). Specifically, it is low level E2 that upregulates early promoter activity (Steger and Corbach, 1997). E2 is believed to achieve this role by interacting with and recruiting cellular transcription factors such as Sp1 (Li et al., 1991) and p300/CBP (Lee et al., 2000a), to the promoter where they can aid transcription. p300/CBP is a transcriptional co-activator with histone acetylase transferase activity and therefore functions by remodelling the chromatin into an 'open' conformation, ready for transcription (Grunstein, 1997).

1.13.4.3 Transcriptional repression

As well as activation of transcription, E2 has the important ability to cause transcriptional repression, and is therefore a crucial regulator of viral gene expression (Bouvard et al., 1994b; Thierry and Yaniv, 1987).

Repression is thought to occur at higher E2 concentrations when E2 has occupied more E2BSs. A likely mechanism is by inhibition of TBP binding the TATA box by steric hindrance when E2 is occupying E2BS#1 (Dostatni et al., 1991). Binding of E2 to E2BS#2 and E2BS#3 may also cause repression by competing with binding of cellular transcription factors such as Sp1 (Demeret et al., 1997; Desaintes et al., 1999). Several

truncated versions of HPV and BPV E2, still containing the C-terminal region, E2C, have been shown to also have transcription repression effects (Bouvard et al., 1994b; Lambert et al., 1987; Liu et al., 1995). The mechanism could either be through forming heterodimers with full-length E2 (Barsoum et al., 1992) or through competition with full-length E2 for E2BSs (Lim et al., 1998).

The importance of the ability of E2 to negatively regulate transcription of the viral oncogenes E6 and E7, is demonstrated by the frequent disruption of the E2 gene through integration in cancers (El Awady et al., 1987; Vernon et al., 1997).

1.13.4.4 Role in replication

As described in 1.13.3, E2 is important for viral DNA replication due to its role in recruiting E1 to the ori, forming the E1-E2-ori complex and therefore initiating assembly of the active E1 helicase (Sedman and Stenlund, 1995). E2-E1 interactions are mediated through the N-terminal TAD of E2 (Benson and Howley, 1995).

However, mutagenesis studies suggest other methods of promoting replication, since a 16E2 mutant that still retained full E1-binding ability, showed reduced replication activity (Sakai et al., 1996). One way BPV-1 E2 may increase replication is to bind RPA, a mechanism also used by E1 (Li and Botchan, 1993). BPV-1 E2 binding to E2BSs has also been shown to upregulate replication of viral DNA that has been transcriptionally repressed through the addition of histones, so E2 can increase replication *in vivo* by overriding inhibitory effects of nucleosomes (Li and Botchan, 1994).

Interestingly, p53 can inhibit HPV replication (Lepik et al., 1998) and E2 can bind p53, but a mutant p53 that cannot bind E2, no longer inhibits replication (Massimi et al., 1999). This suggests a possible role for E2 in restricting the level of viral replication.

1.13.4.5 E2 and apoptosis

E2 has been associated with anti-proliferative and cell-cycle arrest effects (Goodwin et al., 1998; Hwang et al., 1996). The induction of apoptosis by expression of high-risk E2 in has also been well-documented (Blachon et al., 2005; Desaintes et al., 1997; Sanchez-Perez et al., 1997). Whether this apoptosis is p53 dependent or not has been

controversial. p53 independence has been reported from experiments where 18E6 overexpression lowered p53 levels but did not alter apoptosis (Desaintes et al., 1999). p53 dependence has also been reported as a result of inhibiton of 16E2-induced apoptosis when a dominant negative p53 was expressed (Webster et al., 2000). It has been shown that the TAD of E2 is important for apoptosis, but neither transactivation nor replication is necessary for E2-induced apoptosis (Demeret et al., 2003; Desaintes et al., 1999). The current thinking is that E2-mediated apoptosis can be p53 independent and use the caspase pathway, as demonstrated by caspase 8 activation (Blachon et al., 2005; Demeret et al., 2003).

1.13.4.6 Role in viral DNA segregation

Another function of E2, is the ability to bind viral genomes to mitotic host chromosomes, thus ensuring the correct segregation of viral genomes into the daughter cells. This activity has been well characterised for BPV1 E2 (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998) which appears to require binding to the cellular protein Brd4 in order to allow segregation (You et al., 2004). E2 of HPV11, 16 and 18 have also been shown to colocalise to mitotic chromosomes (Van Tine et al., 2004). This function of E2 is likely to contribute to viral genome maintenance and persistent viral infection. 16E2 has also been shown to bind 16E7 and recruit it to mitotic chromosomes at the telophase stage (Gammoh et al., 2006). The significance of this is unclear, but is interesting given the fact that E7 can cause abnormal host genome segregation.

1.13.4.7 Association with E6 and E7

E2 is able to interact directly with both E6 and E7 as described in 1.13.1.7 and 1.13.2.4. The C-terminus of 16E2 is involved in E6 binding (Grm et al., 2005), while the hinge region is important for E7 binding (Gammoh et al., 2006). E6 appears to inhibit the viral DNA replication activity of E2 but enhance viral transcription by E2 (Grm et al., 2005). Most interestingly, E2 has been shown to negatively regulate E6 and E7 independently of its transcriptional repression activity. It can reduce the PDZ-containing protein degradation by E6 (Grm et al., 2005) and can inhibit the transforming activity of E7 possibly by competing with E7 cellular binding partners (Gammoh et al., 2006).

1.13.5 The E5 protein

E5 is a small hydrophobic protein that exists as a dimer in the endoplasmic reticulum (ER), golgi apparatus, plasma membrane and nuclear membrane (Burkhardt et al., 1989; Conrad et al., 1993; Valle and Banks, 1995). The protein is predicted to have three membrane helices and short N- and C-termini (Ullman et al., 1994). In BPV infections, E5 is the major transforming protein (Burkhardt et al., 1987), while E5 of HPV may play a more subtle albeit important role in cancer progression.

1.13.5.1 Effects on extracellular mitogen signalling pathways

BPV1 E5 has been shown to directly bind and stimulate the epidermal growth factor receptor (EGFR) and β platelet-derived growth factor receptor (βPDGFR; Cohen et al., 1993; Goldstein at al., 1994). The main consequence of signalling from these receptors is the activation of genes that cause proliferation, but cellular differentiation can also be triggered (Figure 1.4). HPV16 E5 also enhances EGF-induced signalling (Leechanachai et al., 1992), but whether direct interaction with the EGFR is involved, is uncertain (Conrad et al., 1994; Hwang et al., 1995). One mechanism of increasing receptor signalling, reported for both BPV and HPV, is the association of E5 with a vacuolar H^+ ATPase (Goldstein et al., 1992). This interaction affects the internalisation of the growth factor receptors, promoting recycling of receptors back to the plasma membrane, thus increasing receptor number and prolonging their signalling (Straight et al., 1993). However, the observation that EGFR-activating phosphorylation is also increased by HPV E5, suggests that increased numbers of EGFR due to increased recycling, cannot alone account for the increased signalling (Crusius et al., 1998).

A major signalling pathway activated by EGFRs is the MAPK pathway, and 16E5 has been shown to activate p42/p44MAPK (Crusius et al., 1997). E5-mediated MAPK activation was shown with both EGF and the phorbol ester, PMA, the EGF method being PKC-independent and the PMA method being PKC-dependent (Crusius et al., 1997). E5 also had the ability to activate MAPK in the presence of sorbitol, a chemical that causes osmotic shock. The MAPK, p38, is normally associated with stress responses such as osmotic shock, however, E5 and sorbitol activated only p42/p44MAPK and not p38MAPK (Crusius et al., 2000).

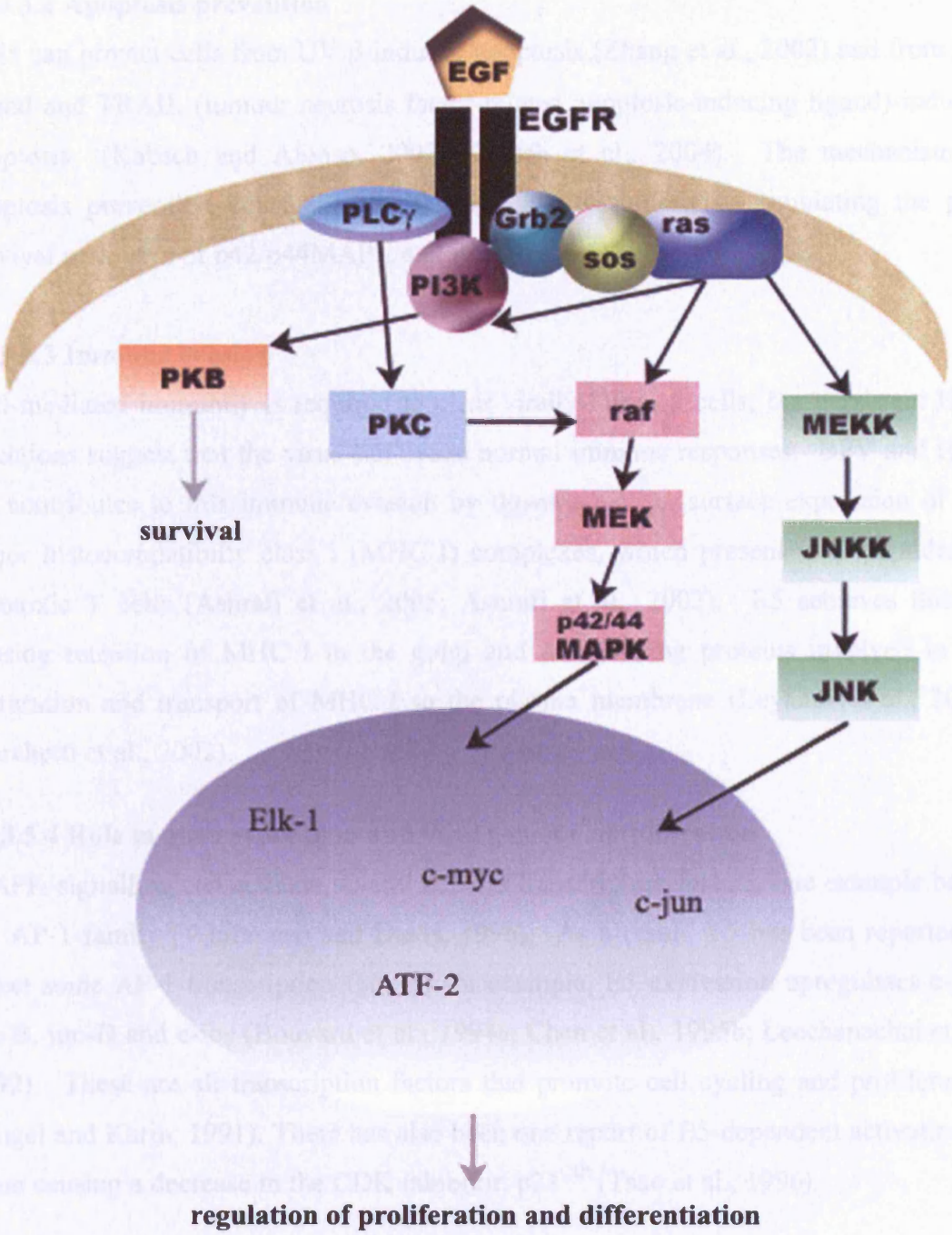


Figure 1.4 Signalling from the EGFR
Activation of the EGFR leads to several signalling cascades including the ras-MAPK pathway, the PLCγ-PKC pathway and the PI3K-PKB pathway. Note that the MAPK pathway can be PKC-dependent or -independent. The PI3K-PKB pathway promotes cell survival and anti-apoptosis responses. The MAPK pathway leads to activation of nuclear transcription factors that promote cell divison, proliferation or differentiation.

1.13.5.2 Apoptosis prevention

16E5 can protect cells from UV β-induced apoptosis (Zhang et al., 2002) and from Fas ligand and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-induced apoptosis (Kabsch and Alonso, 2002; Kabsch et al., 2004). The mechanism of apoptosis prevention appears to be through EGFR signalling, stimulating the pro-survival pathways of p42/p44MAPK and PI3K-PKB (Zhang et al., 2002).

1.13.5.3 Immune evasion

Cell-mediated immunity is required to clear virally-infected cells, but persistent HPV infections suggest that the virus can evade normal immune responses. BPV and HPV E5 contributes to this immune evasion by downregulating surface expression of the major histocompatibilty class I (MHC I) complexes, which present viral peptides to cytotoxic T cells (Ashrafi et al., 2005; Ashrafi et al., 2002). E5 achieves this by causing retention of MHC I in the golgi and deregulating proteins involved in the maturation and transport of MHC I to the plasma membrane (Leykauf et al., 2004; Marchetti et al., 2002).

1.13.5.4 Role in gene expression and viral genome amplification

MAPK signalling can activate several cellular transcription factors, one example being the AP-1 family (Whitmarsh and Davis, 1996). As a result, E5 has been reported to affect some AP-1 transcription factors, for example, E5 expression upregulates c-jun, jun-B, jun-D and c-fos (Bouvard et al., 1994a; Chen et al., 1995b; Leechanachai et al., 1992). These are all transcription factors that promote cell cycling and proliferation (Angel and Karin, 1991). There has also been one report of E5-dependent activation of c-jun causing a decrease in the CDK inhibitor, $p21^{CIP}$ (Tsao et al., 1996).

Several AP-1 binding sites are found in the enhancer region of the viral LCR and AP-1 binding is thought to activate viral gene expression (Chong et al., 1991). Indeed, E5 expression has been shown to upregulate viral genes (Bouvard et al., 1994a), and an HPV31 E5-knockout in raft culture showed a reduction in viral transcripts from the late promoter and reduced levels of E1^E4 protein (Fehrmann et al., 2003).

As well as affecting viral gene expression, E5-knockouts appeared to affect viral genome amplification. An HPV31 E5-knockout in raft culture, had decreased viral genome amplification (Fehrmann et al., 2003). An HPV16 E5-knockout in raft culture,

had more subtle effects; ~2-fold decrease in cellular DNA synthesis in the suprabasal layer, but no observed difference in viral genome amplification (Genther et al., 2003).

1.13.6 The capsid proteins; L1 and L2

L1 is the major capsid protein present at 360 copies per virus, and L2 is the minor capsid protein present at ~12 copies (Finnen et al., 2003). Both proteins have NLSs in their C-termini (Nelson et al., 2000; Sun et al., 1995).

1.13.6.1 Encapsidation of the viral genome

Viral DNA replication is thought to occur in nuclear compartments called ND10s, and L2 functions by entering ND10s, associating with the viral genome and recruiting L1 to the site (Day et al., 1998; Florin et al., 2002b). As well as encapsidating the viral genome, L2 may also have a role in directing the viral genome to the nucleus upon viral entry, therefore encouraging viral transcription (Day et al., 2004). L2 appears to reorganise ND10 domains, for example it causes the release of an ND10 protein, Sp100, before L1 is recruited (Florin et al., 2002b).

1.13.6.2 Assembly of the capsid

The major protein component of ND10 is the promyelocytic leukaemia protein (PML), and although this is considered to be important for papillomavirus infection (Day et al., 2004), it appears that it is unnecessary for assembly of L1 and L2 into VLPs (Becker et al., 2004). L1 alone, or L1 together with L2, can self-assemble into VLPs *in vitro* and in cells (Hagensee et al., 1993; Rose et al., 1993; Zhou et al., 1991). VLPs are well-documented, as a result of their use as prophylactic vaccines (see 1.8.3). L1 molecules associate by disulphide bonding to form pentameric capsomeres, to which a single L2 can bind through hydrophobic interactions involving sequences from its N- and C-termini (Finnen et al., 2003; Okun et al., 2001). Reducing conditions are required, although in non-reducing conditions, L1 capsomere formation can be enhanced by the presence of L2 (Ishii et al., 2005).

1.13.7 The E1^E4 protein

E1^E4 is translated from a spliced mRNA, with the first five amino acids of the protein being encoded by the E1 ORF, while the rest are encoded by the E4 ORF, hence the name 'E1^E4' (Doorbar et al., 1988; Doorbar et al., 1990; Nasseri et al., 1987). E1^E4 proteins are small (for example 16E1^E4 is 10 kDa; Figure 1.5), unusually rich in proline and charged residues and many have a N-terminal conserved leucine cluster, for example LLKLL for 16E1^E4 and LLNLL 11E1^E4 (Doorbar and Myers, 1996). Generally, the protein sequence varies between different HPV types, but there is some degree of conservation in the C-terminal region within cutaneous types and within mucosal types. As a result, it has been proposed that E1^E4 may play a role in determining tissue specificity (Smith and Campo, 1985). There are exceptions, however, since HPV1 and HPV2 are predominantly cutaneous viruses (although HPV2 is occassionally found in mucosal sites), but their E1^E4 sequences are not well-matched to sequences of other cutaneous types (Doorbar et al., 1989).

1.13.7.1 Oligomerisation and post-translational modificiations

The C-terminal region has been shown to be important for dimer and oligomer formation for 1E1^E4 (Ashmole, 1998), 11E1^E4 (Bryan et al., 1998) and 16E1^E4 (Roberts et al., 1997; Wang et al., 2004). Full-length 16E1^E4 was shown to exist as monomers, dimers and hexamers, while a Δ87-92 16E1^E4 was only detected as a monomer (Wang et al., 2004). When SDS-PAGE is performed in non-reducing conditions, several high molecular weight species of 16E1^E4 are seen on the gel, however, the presence of reducing agents or the mutation of the two cysteines (C61 and C62) to alanines prevents this observation (Roberts et al., 1997; Wang et al., 2004). This suggests that the oligomers are stabilised by disuphide bonding.

E1^E4 proteins can be post-translationally modified by phosphorylation and by N-terminal proteolysis. E1^E4 proteins known to be phosphorylated are 1E1^E4 (Grand et al., 1989) and 11E1^E4 (Bryan et al., 2000) and there has been a report of a phosphorylated form of 16E1^E4 (Wang et al., 2004). The findings of these papers are further discussed in 1.15.5.

N-terminally cleaved forms of E1^E4 have been well-characterised for HPV1. In HPV1 lesions, full-length (17 kDa) E1^E4 is more abundant in the lower layers, while

Figure 1.5 16E1^E4 amino acid sequence and functional domains
16E1^E4 has 92 amino acids, the first five of which are encoded by the E1 ORF, while the remaining are encoded by the E4 ORF. The main functional domains identified so far include, the keratin binding motif, a proline-rich region important for cell cycle arrest, and a C-terminal domain involved in keratin collapse and oligomerisation.

the truncated forms (16 kDa, 11 kDa and 10 kDa) are more abundant in the upper layers, suggesting that this proteolysis is differentiation-dependent (Breitburd et al., 1987; Doorbar et al., 1988). Recently, different activities have been assigned to the different forms of 1E1^E4 (Knight et al., 2004: see 1.13.7.4).

An N-terminally truncated form of 16E1^E4 has also been proposed. 16E1^E4 staining with an antibody that recognises a central epitope, often reveals small perinuclear bundles correlating with E1^E4 and collapsed keratin filaments (see 1.14.7.3 and 1.14.7.4), but when an N-terminal-specific antibody was used, only the periphery of the bundles were stained (Doorbar et al., 1997). This differential staining suggests that there is a form of 16E1^E4 lacking the N-terminus and that this may behave differently to the full-length protein.

1.13.7.2 E1^E4 expression patterns

In a productive infection E1^E4 is expressed at high levels, particularly in the intermediate and superficial layers (Crum et al., 1990; Doorbar et al., 1997; Middleton, 2003; Stoler et al., 1989). In some infections, for example with HPV1, E1^E4 constitutes up to 30 % of the total protein in the lesion (Doorbar et al., 1986). In malignancy, however, integration events frequently lead to disruption of the E1^E4 ORF, resulting in reduction or complete loss of E1^E4 expression (Durst et al., 1992; Middleton, 2003). The high level expression requires activation of the differentiation-dependent promoter (Hummel et al., 1992), coincides with the onset of viral genome amplification (Breitburd et al., 1987; Doorbar et al., 1997; Peh et al., 2002) and occurs prior to L1 expression (Doorbar et al., 1997; Middleton, 2003).

Although, E1^E4 levels per cell can become very high (as seen with immunostaining), the protein is apparent in only a subset of infected cells in both cell culture systems and rafts (Frattini et al., 1997; Genther et al., 2003) and in lesions (Doorbar et al., 1989; Nicholls et al., 2001). There is growing evidence for E1^E4 being present at low levels but perhaps difficult to detect by immunostaining, so there is the possibility that E1^E4 is expressed at low level in many of these cells that appear E1^E4–negative. For example, E1^E4-encoding transcripts are detected in undifferentiated monolayer cells (Doorbar et al., 1990; Hummel et al., 1992) and even in the basal cells of an HPV6 infection (Stoler et al., 1989). More recently, there have been reports on E1^E4 disturbing keratinocyte differentiation and its role in viral DNA replication, suggesting

that E1^E4 functions prior to activation of the differentiation-dependent promoter (see 1.13.7.6 and 1.13.7.7). Nakahara et al. (2005) also reported that by Western blotting, 16E1^E4 could be detected in monolayer cells and rafts, whereas, by immunostaining, it was only detected in the terminally differentiated layers. In these rafts, effects of E1^E4, such as increased DNA synthesis, could be detected earlier (i.e. at lower layers), before E1^E4 itself could actually be detected (by immunostaining).

E1^E4 of HPVs are predominantly cytoplasmic. The E1^E4 of certain cutaneous types are often stained as cytoplasmic inclusion granules (Doorbar et al., 1989; Egawa, 1994), but filamentous E1^E4 (Roberts et al., 1993) and nuclear E1^E4 has been described for HPV1 (Roberts et al., 2003). Lesions caused by the animal papillomaviruses ROPV (rabbit oral papillomavirus) also have predominantly cytoplasmic E1^E4, whereas CRPV and COPV (canine oral papillomavirus) infections have cytoplasmic and nuclear E1^E4 (Peh et al., 2002).

Mucosal/anogenital HPV types typically have a filamentous pattern for E1^E4 due to their association with keratin (see 1.13.7.3; Doorbar, 1991; Sterling et al., 1993). As well as showing a filamentous phenotype, 16E1^E4 (Doorbar et al., 1991) and 31E1^E4 (Pray and Laimins, 1995) but not 1E1^E4 (Roberts et al., 1993) appear to cause filament collapse and the E1^E4 localises as a perinuclear aggregate or bundle. 11E1^E4 predominantly appears as perinuclear aggregates (Bryan et al., 1998) but a filamentous distribution has also been described (Bryan et al., 2000). In addition, 11E1^E4 has been shown to associate with the cornified envelope of differentiated keratinocytes (Bryan et al., 1998).

1.13.7.3 Association with keratins

The filamentous pattern seen with HPV16 E1^E4 can be attributed to its association with keratin filaments. Keratins 8 and 18 were co-purified with 16E1^E4 from cultured epithelial cells when an immunoprecipitation was carried out with an anti-16E1^E4 antibody (Doorbar et al., 1991). Similarly, keratins 8 and 18, immunoprecipitated using anti-keratin 8/18 rabbit serum, were shown to bind bacterially expressed 16E1^E4 (Wang et al., 2004). The association was shown to be direct by a far-Western experiment where SDS-PAGE-separated 16E1^E4 was transferred to a membrane and then shown to bind purified keratin 18. Also, when 16E1^E4 was incubated with

purified keratins 8 and 18 that were loaded on a membrane, 16E1^E4 binding to keratin 18 was easily detected, whereas binding to keratin 8 appeared to be weaker (Wang et al., 2004).

The binding of 16E1^E4 to keratin appears to affect normal keratin dynamics by keeping keratin in the insoluble fraction of the cell, despite phosphorylation of keratin which normally leads to its solubilisation (Wang et al., 2004). The 16E1^E4-mediated collapse or reorganisation of keratin filaments has also been described, both in cultured cells (Doorbar et al., 1991), and *in vivo* (Doorbar et al., 1997; Wang et al., 2004).

The N-terminal 16 residues of 16E1^E4 is sufficient to target green fluorescent protein (GFP) to keratin (Wang et al., 2004). However, mutation of the leucine cluster region, for example, LLGLL (residues 10-14) of 1E1^E4 or LLKLL (residues 12-16) of 16E1^E4, can prevent colocalisation with keratin (Roberts et al., 1994a; Roberts et al., 1997). The collapse of keratin requires the C-terminal region of E1^E4 (Roberts et al., 1997) and as discussed in 1.13.7.1, this region is also important for oligomerisation. Interestingly, a Δ87-92 16E1^E4 oligomerisation mutant, colocalises partially with keratin (as seen with immunostaining) but is soluble and cannot inhibit keratin dynamics (Wang et al., 2004). A model of E1^E4/keratin dynamics has therefore been proposed by Wang et al. (2004) whereby E1^E4 molecules are bound to keratin but also bound to other E1^E4 molecules through their C-termini, therefore cross-linking the keratins, preventing their solubilisation. The purpose of disrupting keratin dynamics or causing keratin collapse could be to weaken cell integrity and aid virus release (see 1.13.7.8).

1.13.7.4 Association with cyclins and cell cycle effects

16E1^E4, 11E1^E4 (Davy et al., 2002), 18E1^E4 (Nakahara et al., 2002) and 1E1^E4 (Knight et al., 2004) have all been shown to cause cells to arrest in the G_2 phase of the cell cycle. With 16E1^E4, when two threonines in the proline-rich region were mutated to alanines (T22A T23A) and expressed in mammalian cells, cell cycle arrest was abrogated (Davy et al., 2002). In the yeast, *S pombe,* a T23A mutant was expressed and this mutation was sufficient for blocking arrest. To investigate whether phosphorylation of this threonine was important for arrest (T23 is a putative CDK1 phosphorylation site), it was replaced with aspartic or glutamic acid (T23D and T23E), both of which can mimic phosphate, however neither mimic caused G_2 arrest (Davy et

al., 2002). 16E1^E4 was subsequently found to colocalise with cyclin B/CDK1 and sequester it in the cytoplasm and this association was absent in a T22A T23A 16E1^E4 mutant (Davy et al., 2005). Active cyclin B/CDK1 is required to enter the nucleus to trigger mitosis (Smits and Medema, 2001), and it appears that the mechanism of 16E1^E4-induced arrest is via the inhibition of cyclin B nuclear entry.

Interestingly, cell cycle arrest by HPV1 is mediated by the 16 kDa truncated protein and not the full-length 17 kDa protein, although coexpression of both did not inhibit the arrest (Knight et al., 2004). The G_2 arrest is again dependent on a threonine residue in the proline-rich region (T13) and can be reversed by exogenous expression of cyclin B from a cytomegalovirus (CMV) promoter.

Recently, cyclin A/CDK2 has also been found to associate with 16E1^E4 (Davy et al., 2006). The function of this interaction is also likely to be related to cell cycle arrest. The ability to cause cell cycle arrest could be the mechanism by which E1^E4 acts as an enhancer of viral DNA replication (see 1.14.7.6). This is because a HPV-infected cell arrested at G_2 and expressing E7, would possess a sustained S-phase-like state, allowing the virus to use the cellular DNA replication machinery for its own replication.

1.13.7.5 Other cellular binding partners

A yeast-two hybrid screen of 16E1^E4 with an epithelial cell cDNA library identified a novel DEAD box protein as a E1^E4 binding partner (Doorbar et al., 2000a). Dead box proteins possess the motif, apartic acid-glutamic acid-alanine-aspartic acid, and have ATP and RNA binding sites. They play a role in RNA processing, stability and biogenesis of ribosomes (Tanner and Linder, 2001). This novel DEAD box protein was named E1^E4-DEAD box protein (E4-DBP). The association was found to require the C-terminal region of 16E1^E4. A putative role of 16E1^E4 in RNA stability/translation has therefore been proposed, and this could be important in light of the finding that 31E1^E4 is important for production of late viral transcripts (see 1.13.7.8).

E1^E4 of HPV1 was found to bind zinc and this association was dependent on the histidine residues of the protein (Roberts et al., 1994b). Dimerisation and keratin

colocalisation activities were not affected in a mutant unable to bind zinc, so the function of zinc binding is unknown.

1.13.7.6 Role in viral DNA amplification

Consistent with the observation that E1^E4 expression correlates with viral genome amplification, there have been several reports of E1^E4 being necessary for viral genome amplification. The first report was of CRPV that was mutated to abolish E1^E4 expression (Peh et al., 2004). In an infection with this mutant, papillomas were produced but viral genome amplification did not occur. More recently, HPV31 (Wilson et al., 2005) and HPV16 (Nakahara et al., 2005), both with mutated E1^E4, were grown in raft culture. Both mutants revealed a significant reduction in cellular DNA synthesis in the suprabasal layers and a reduction in viral genome amplification. For HPV16, the LLKLL leucine cluster of E1^E4 was shown to be necessary for viral DNA replication (Nakahara et al., 2005).

1.13.7.7 Effects on keratinocyte differentiation

Keratinocyte differentiation is tightly regulated in the epithelium and aspects of the papillomavirus life cycle are dependent on this differentiation as previously discussed. The expression of E1^E4, however, appears to disturb normal differentiation as shown by the absence or reduction of some differentiation-specific cellular proteins. For example, filaggrin, loricrin, and keratins 1 and 10 expression is reduced in 1E1^E4-positive cells as compared to neighbouring E1^E4-negative cells (Doorbar et al., 1997). Similarly, 16E1^E4 expression correlated with lowered keratins 4 and 13 (Doorbar et al., 1997) and 11E1^E4 caused a reduction in loricrin and keratin 10 in the cornified cell envelope (Brown and Bryan, 2000). This phenomenon was also observed in raft cultures, where 16E1^E4 coincided with keratin 10-negative cells in the upper epithelial layers (Nakahara et al., 2005).

1.13.7.8 Role in virus production and release

There is now strong evidence that E1^E4 is necessary for virus production given its role in late viral events, such as viral genome amplification (Nakahara et al., 2005; Wilson et al., 2005). It also appears to be important for expression of late viral proteins, particularly in CRPV infections, where without E1^E4, L1 was no longer expressed (Peh et al., 2004). Similarly, Wilson et al. (2005) reported a reduction in

late viral transcripts when E1^E4 was absent in HPV31-containing differentiated keratinocytes.

In addition to aiding virus production, E1^E4 is thought to contribute to virus release by increasing the fragility of keratinocytes in the uppermost epithelial layers. This hypothesis arises from knowledge of the activities of E1^E4, for example, its ability to disrupt keratin dynamics (Wang et al., 2004) and contribute to the formation of abnormal cornified cell envelopes (Brown and Bryan, 2000). 16E1^E4 has also been shown to cause apoptosis when overexpressed in cells (Raj et al., 2004). The mechanism may be related to its association with mitochondria at late time points following keratin collapse. The association with mitochondria was found to be dependent on the leucine cluster sequence (LLKLL). The *in vivo* significance of apoptosis induction is unclear, however, apoptosis of epithelial cells may be another way to aid virus release and improve transmission.

1.14 Protein phosphorylation

Protein phosphorylation by protein kinases is one of the most frequent post-translational protein modifications and can have dramatic affects on protein function. The cell therefore uses phosphorylation (by kinases) and dephosphorylation (by phosphatases) to regulate many signalling pathways and other important cellular activities (Hardie, 1999). The most common form of phosphorylation is the formation of phosphate esters of the amino acids, serine, threonine and tyrosine (Pawson, 1994; Taylor et al., 1995). It is thought that at any time, 30 % of a cell's proteins are phosphorylated, some at multiple sites (Zolnierowicz and Bollen, 2000).

The enzymatic transfer of phosphate to a protein was first discovered in 1954 when liver extract was found to phosphorylate casein (Burnett and Kennedy, 1954). A year later the first functional role of phosphorylation was discovered when phosphorylation was found to regulate the activity of the enzyme, glycogen phosphorylase (Fischer and Krebs, 1955; Sutherland and Wosilait, 1955). The importance of this work was later recognised when Fischer and Krebs were awarded the 1992 Nobel Prize in Physiology or Medicine for their 'discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism.'

With the completion of the human genome project, it is now believed that around 1.7 % of all human genes encode protein kinases, bringing the number to approximately 500 protein kinases (Manning et al., 2002). Of these, 164 map to chromosomal loci associated with tumours and another 80 map to loci associated with other diseases. This emphasises the role of phosphorylation in regulating cellular activities, and reveals why kinases and phosphatases make attractive targets for therapeutics.

1.15 Phosphorylation of papillomavirus proteins

1.15.1 Phosphorylation of E6

PKA has been shown to phosphorylate E6 at a site within the PDZ binding domain (Kuhne et al., 2000). Interestingly, this has been shown to occur with only high-risk E6 proteins and not low-risk ones. Phosphorylation inhibits the interaction of E6 with hDlg and prevents E6-mediated degradation of hDlg.

1.15.2 Phosphorylation of E7

E7 was shown to be labelled with ^{32}P, however mutating serine 31 or serine 71 appeared to decrease this (Storey et al., 1990). Subsequently, both serines 31 and 32 were found to be phosphorylated by casein kinase II (CKII; Barbosa et al., 1990). Mutation of residues 31 and 32 to arginine and proline respectively, did not alter pRB binding, but did decrease anchorage independent growth of mouse fibroblasts. It was later shown that phosphorylation by CKII at serines 31 and 32 increases the affinity of E7 for TBP (Massimi et al., 1996). The original observation of serine 71 being a phosphorylation site was confirmed by Massimi and Banks (2000). It was found that E7 is differentially phosphorylated during the cell cycle, with serine 71 being phosphorylated at S-phase by a yet unknown kinase, and CKII phosphorylating serines 31 and 32 at an earlier cell cycle stage (Massimi and Banks, 2000).

1.15.3 Phosphorylation of E1

BPV1 E1 was identified as a phosphoprotein due to its incorporation of ^{32}P (Sun et al., 1990). Several phosphorylation sites in BPV1 E1 have since been described. The first to be identified was threonine 102 which was shown to be phosphorylated by CDK1 (Lentz et al., 1993). Also, serine 109 was found to be phosphorylated *in vivo* and then shown to be phosphorylated *in vitro* by protein kinase A (PKA) and protein kinase C (PKC; Zanardi et al., 1997). Mutation of serine 109 to alanine causes an improvement in viral DNA replication and ori binding, while mutating to glutamic acid to mimic phosphorylation decreases replication. Another phosphorylation event, at serine 48 was found to affect replication activity positively (McShan and Wilson, 2000). Serine 48 was identified as a phosphorylation site of CKII and mutating to glycine lead to a loss in replication activity, while mutating to aspartic or glutamic acid restored activity. Finally, serine 584, a residue conserved in many E1 proteins, was identified as a phosphorylation site of casein kinase I and II (CKI and CKII) although the function is unclear (Lentz, 2002).

A role for E1 phosphorylation in HPV has also been investigated. Mutation of candidate CDK phosphorylation sites of HPV11 E1 was shown to impair viral replication (Ma et al., 1999). It was later found that CDK (probably CDK2) phosphorylation can inhibit the export of E1 from the nucleus (Deng et al., 2004). Mutating either of the target serines (S89, 93, 107) to alanine, inhibited nuclear localisation and also reduced replication.

1.15.4 Phosphorylation of E2

The major phosphorylation sites of BPV1 E2 have been mapped to serines 298 and 301 (McBride et al., 1989) and mutation of serine 301 to alanine causes ~20-fold increase in viral DNA plasmid copy number (McBride and Howley, 1991). This may relect the preference of E1 to bind non- or under-phosphorylated E2 (Lusky and Fontane, 1991) or the fact that serine 301 phosphorylation increases ubiquitination and degradation of E2 (Penrose and McBride, 2000). Phosphorylation of BPV1 E2 may also affect its ability to segregate viral genomes by binding to mitotic chromosomes. Additional phosphorylated serines were identified and it was found that a phosphorylation mutant with four serines mutated (serines 235, 290, 298 and 301; all in the central hinge

region) could no longer bind mitotic chromosomes (Lehman and Botchan, 1998). In this case, serine 235 was considered critical, as a mutant with just the other three serines mutated still retained the ability to colocalise with chromosomes and segregate viral DNA.

1.15.5 Phosphorylation of E1^E4

Phosphorylation of E1^E4 was first reported by Breitburd et al. (1987). HPV1 E1^E4 proteins (full-length and truncated) from warts were analysed by two-dimensional gel electrophoresis (2D SDS-PAGE). Each E1^E4 protein had several isoelectric variants meaning the variants were differentially charged, and interestingly, the most superficial wart layer had more acidic variants than the deeper layers did. The more acidic variants of the 16 kDa and 17 kDa become more intensely labelled with ^{32}P than the more basic variants. Taken together, the results suggest that 1E1^E4 is phosphorylated *in vivo* and phosphorylation increases as the protein reaches more superficial layers. 1E1^E4 phosphorylation was further analysed by Grand et al. (1989). The 16kD and 17kD isoforms were subsequently shown to be phosphorylated *in vitro* by PKA (Grand et al., 1989).

11E1^E4 phosphorylation has also been described and some phosphorylation sites mapped (Bryan et al., 2000). When HPV11-infected human genital tissue was grown as a xenograft in a mouse and radiolabelled with ^{32}P, the E1^E4 became labelled. *In vitro* kinase assays also demonstrated that 11E1^E4 can be phosphorylated by p42MAPK at threonine 53 (within a variant of the MAPK consensus site) and by PKA at threonine 36 (within a PKA consensus site) and serine 44 (within a variant of the PKA consensus site; Figure 1.6). Bryan et al. (2000) also made phosphorylation site-mutant 11E1^E4s and reported that the MAPK site-deficient E1^E4 had a reduced filamentous distribution as compared to wild type E1^E4. Wild type 11E1^E4 has been shown to oligomerise (Bryan et al., 1998) but this MAPK mutant E1^E4 did not colocalise with wild type E1^E4. The PKA mutant 11E1^E4 had similar cellular distribution to the wild type and also colocalised with wild type E1^E4, suggesting it still retained the ability to oligomerise.

16E1^E4 phosphorylation has been reported by Wang et al. (2004) but not discussed in

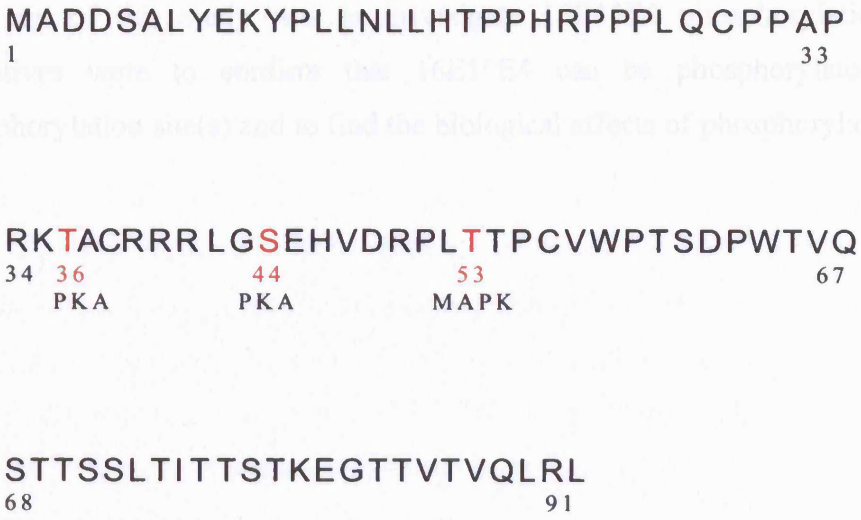


Figure 1.6 Phosphorylation sites of 11E1^E4
The HPV11 E1^E4 amino acid sequence is shown together with phosphorylation sites (red) reported by Bryan et al. (2000).

any detail. Considering that other E1^E4 types are phosphorylated and functional roles for HPV protein phosphorylation have been found, it was decided to focus further on 16E1^E4 phosphorylation.

The aim of this study was to investigate 16E1^E4 phosphorylation. The main objectives were to confirm that 16E1^E4 can be phosphorylated, to map the phosphorylation site(s) and to find the biological effects of phosphorylation.

Chapter 2: Materials and Methods

2.1 Suppliers of reagents

Except where specified, including below, reagents were obtained from Sigma-Aldrich Company Ltd. (UK), BDH Laboratory Supplies (UK) or Fisher Scientific (UK).

Suppliers of commonly used reagents are listed below:
Agarose, ammonium persulfate (APS), ethidium bromide, sodium dodecyl sulfate (SDS) and N,N,N,N'-tetra-methyl-ethylenediamine (TEMED) were obtained from Bio-Rad (UK). Ultra Pure ProtoGel Acrylamide was obtained from National Diagnostics (UK). Benzonase® was obtained from Novagen, Merck (UK). Complete Protease Inhibitor Cocktail Tablets were obtained from Roche (UK). Marvel was obtained from Premier International Foods (UK). Fetal calf serum (FCS) was obtained from Perbio Science Ltd. (UK). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (UK). All primers were produced by Sigma-Genosys (UK).

2.2 Components of commonly used buffers and reagents

Commonly used buffers were prepared according to Table 2.1.

Table 2.1 Buffers and reagents

2 x Laemmli's buffer	4 % (w/v) SDS, 20 % (v/v) glycerol, 0.1 M DTT, 0.12 M Tris, pH 6.8, containing a trace of bromophenol blue
10 x loading buffer	50 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue, 0.4 % (w/v) xylene cyanol
LB broth	1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 86 mM NaCl
LB agar	LB broth + 2 % (w/v) Bacto agar
PBS	0.17 M NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4
TBE	0.1 M Tris base, 0.62 % (w/v) boric acid, 5 mM EDTA, pH 8.0
TBS	0.15 M NaCl, 10 mM Tris pH 7.5
trypsin-versene	0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.27 mM EDTA, 0.13 % (w/v) trypsin, 0.001 % (w/v) phenol red, pH 7.6-7.8
pen/strep stock (cell culture antibiotics)	0.6 % (v/v) penicillin, 1 % (v/v) streptomycin
SDS electrophoresis buffer	25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS
transfer buffer	30 mM Tris base, 240 mM glycine, 20 % (v/v) methanol

2.3 Cell culture methods

2.3.1 Cell lines

SiHa cells were used for most of the cell culture experiments. These cells are an adherent cervical carcinoma cell line containing integrated HPV16 DNA (Friedl et al., 1970). SiHa cells constitutively express E6/E7 but lack E1^E4 expression due to loss of the E4 ORF. This cell type gives high efficiency infection with recombinant adenoviruses (rAds). C33A, NIKS and COS-7 cell lines were also used. C33A cells are a cervical epithelial carcinoma cell line that is not HPV-transformed. C33A cells lack a functional p53 gene due to an inactivating mutation at codon 273 (Scheffner et al., 1991). NIKS are a HPV-negative spontaneously immortalised human keratinocyte cell line (Allen-Hoffmann et al., 2000) and COS-7 cells are a simian virus 40 (SV40) transformed, African green monkey kidney cell line (Gluzman, 1981).

2.3.2 Maintenance of cells

Cells were maintained in 75 cm^2 Corning flasks in 10 ml of DMEM (Dulbecco's modified Eagle's Medium) supplemented with 10 % (v/v) fetal calf serum (FCS) and 1 % (v/v) antibiotics stock (pen/strep). In this study, the concentrations of FCS and pen/strep were kept constant at 10 % and 1 % respectively, unless otherwise stated, and DMEM supplemented with these reagents is termed 'complete DMEM'. Cells were incubated at 37 °C in a 5 % CO_2 environment. When the cells reached confluency, they were passaged by seeding 10 % of them into a new flask (approximately twice a week). To detach the adherent cells from the flask, they were washed with phosphate buffered saline (PBS), incubated for 5 min at 37 °C with trypsin-versene until they detached from the bottom of the flask and then suspended in more complete DMEM. When cells were required to be seeded at a particular density, the cells in suspension were counted on a haemocytometer, using a phase contrast light microscope (Nikon Eclipse TS100, Surrey, UK) to estimate the cell concentration.

2.3.3 Long-term storage of cells

Cells were cultured until confluent and then a suspension of ~$1x10^6$ cells was prepared

as above (using trypsin-versene). They were then pelleted (2500 g, 5 min) and then resuspended in 1 ml of freezing medium (90 % (v/v) FCS, 10 % (v/v) dimethyl sulphoxide (DMSO)) and transferred to a cryogenic vial. This was stored at -80 °C overnight, wrapped in 12-15 layers of tissue paper to slow down the freezing of the cells, before being transferred to liquid nitrogen. When required, the cells were rapidly defrosted at 37 °C and added to 10 ml of complete DMEM. The cells were pelleted as above, resuspended in 10 ml of complete DMEM and transferred to a 75 cm^2 flask.

2.3.4 TVG402 monoclonal antibody production

TVG402 was produced from a hybridoma cell line (Doorbar et al., 1992), which was maintained in complete DMEM. Following the recovery of the TVG402 hybridoma cell line from long-term storage, the cells were passaged at least six times before being used for antibody production. The cells were then grown until they were over-confluent (~4-7 days after confluence was reached). Subsequently, the supernatant containing ~20-50 μg/ml of antibody was collected and then centrifuged to remove cell debris (1000 g, 10 min). The cell culture supernatant was then filtered through a 0.2 μm filter, sodium azide added to 0.02 % (v/v), and then the antibody stock was stored at 4 °C.

2.3.5 Infection with recombinant adenovirus (rAd)

Recombinant adenovirus preparations were produced by Dr Deb Jackson (NIMR). SiHa cells were seeded in 90 mm Nunc culture dishes in 5 ml of complete DMEM at a concentration of $5x10^5$ cells per dish. After 24 h, 16E1^E4-expressing adenovirus (rAdE1^E4) or βGAL-expressing adenovirus (rAdβGAL) was added to the media at a 100 MOI (multiplicity of infection) for each 90 mm dish. Cells were harvested after 24 h of incubation with the rAds unless otherwise stated.

2.3.6 Transient transfections

Plasmid DNA purified with the HiSpeed Maxi Kit (Qiagen, UK), was used for

transfection of cultured cells. Transfection was performed using the Effectene® Transfection Kit (Qiagen, UK) according to the manufacturer's protocol. Cells were seeded at the densities shown in Table 2.2, 24 h prior to transfection. Medium containing the transfection reagent was removed from the dishes/wells 6 h following transfection and replaced with fresh complete DMEM. Cells were harvested 24 h post-transfection (18 h following renewal of the medium) unless otherwise stated.

Table 2.2 Cell seeding densities for transfections

Culture format	Number of cells to seed
24-well plate	3×10^4 per well
6-well plate	2×10^5 per well
60 mm dish	3×10^5 per dish

2.3.7 Stable transfection of an HPV16 E5-expressing vector

SiHa cells were stably transfected with an E5-expressing vector by Dr Qian Wang (NIMR). Briefly, cells were co-transfected with the HPV16 E5-expressing vector, pMT3H16E5KC (a kind gift from Dr Dan DiMiao, Department of Genetics, Yale University School of Medicine, CT, USA) and pBabe-puro (Morgenstern and Land, 1990) using Effectene®. After selection by G418, HPV16 E5-positive clones were detected by RT-PCR (see 2.5.11).

2.3.8 Okadaic acid (OA) experiments

OA was dissolved in ethanol to make a 50 μg/ml stock for storage at –20 °C. It was either added to the cell culture media 1 h before harvesting the cells or included in the SDS cell lysis buffer, at a concentration of 1 μg/ml.

2.3.9 MAPK inhibitor experiment

SiHa cells were grown in 90 mm dishes and infected with rAdE1^E4 (see 2.3.5). Six h

post-infection, the cell culture medium was removed and replaced with 5 ml of complete DMEM. MAPK inhibitors dissolved in DMSO or 10 µl DMSO (as the negative control) were then added to the media. The inhibitors used were, the p38MAPK inhibitor, SB 203580 (Calbiochem, UK) at a concentration of 5 µM, and the MEK inhibitors, PD 98059 (Calbiochem, UK) at a concentration of 50 µM and U 1026 (Calbiochem, UK) at a concentration of 5 µM. The cells were harvested after 18 h incubation.

2.3.10 CDK1/2 inhibitor experiment

SiHa cells were grown in 90 mm dishes and infected with rAdE1^E4 (see 2.3.5). Six hours post-infection, the virus-containing medium was removed and replaced with 5 ml of complete DMEM. The CDK1/2 inhibitor, Roscovitine (dissolved in DMSO), was then added to a final concentration of 30 µM and for the negative control, 10 µl of DMSO was added. The cells were harvested after 18 h incubation.

2.3.11 Harvesting cells for protein analysis

Cultured cells growing in plates/dishes (90 mm dishes, 60 mm dishes or 6-well plates) were washed with PBS, then 1.5 ml of PBS containing 1 mM EDTA was added (per well/dish) and left for 5 min at R/T. The cells were then scraped using Corning cell scrapers, collected into 1.5 ml microfuge tubes and centriguged at 1000 g for 5 min to pellet the cells. The supernatant was carefully pipetted and discarded and the cell pellet used immediately or frozen at –80 ° C.

2.3.12 Fixing and blocking cells for immunostaining

When cells were cultured for immunostaining, they were grown on glass coverslips (13 mm diameter) in dishes/plates. When ready to be fixed, the cell culture medium was removed and the cells washed three times with PBS. The cells were then fixed with ice-cold methanol for 2 min. Following this, the cells were washed twice with PBS then incubated at 4 °C overnight with 2 % (w/v) bovine serum albumin (BSA) in PBS

containing 0.01 % (v/v) sodium azide. The cells were stored for longer periods at 4 °C in this blocking solution if necessary.

2.4 Cell staining and analysis

2.4.1 Immunostaining

Table 2.3 shows a list of primary antibodies used in immunostaining. Cells previously treated with a solution of BSA (see 2.3.12) were incubated with primary unconjugated antibodies diluted in 0.1 % (w/v) FCS in PBS, at 37 °C for 1 h. They were then washed five times in PBS at 5 min intervals. Following this, the cells were incubated with Alexa Fluor® 488- or Alexa Fluor® 594-conjugated secondary antibodies (Molecular Probes, UK; diluted 1:200) and 1 μg/ml of 4',6-diamino-2-phenylindole (DAPI) diluted in 0.1 % (v/v) FCS in PBS, at 37 °C in the dark for 1 hr. When Alexa Fluor® 488-conjugated TVG405, a mouse anti-16E1^E4 antibody, was used, it was added to the cells at the same time as the secondary antibodies and DAPI. Finally, the coverslips were washed five times in PBS as above, rinsed briefly with ddH_20 then inverted onto 4 μl of citifluor (Agar Scientific, UK) on a microscope slide.

Table 2.3 Primary antibodies for immunostaining

Target Protein/ Epitope	Primary Antibody	Dilution	Source / Reference
16E1^E4 epitopes recognised: PKPSPWAP KPSPWAPK PSPWAPKK SPWAPKKH PWAPKKHR	Alexa Fluor® 488-conjugated TVG405 (human Fab)	1:75	(Doorbar et al., 1997)
Keratin	anti-pan-keratin, c2562 (mouse monoclonal)	1:200	Sigma, UK
Cyclin A	6E6 (mouse monoclonal)	1:400	Novocastra, UK

2.4.2 Microscopy

Cells were observed on a fluorescent Labophot II microscope (Nikon, Kingston upon-Thames, UK). Digital images were captured with a SenSys monochrome camera and IP Lab imaging software (Roper Scientific, Marlow, UK). Fluorescent signals were overlaid by computer assistance.

2.5 DNA methods

2.5.1 DNA constructs used

Bacterially-expressed wild-type C-terminus hexa-histidine tagged 16E1^E4 with a 'leucine, glutamic acid' linker (WT His-E1^E4) was produced from the pET-28(b)+ expression vector (Novagen, UK). The construct was produced by Dr Pauline McIntosh (NIMR). The forward primer was GGG CCC TCG GTT TCC A_1TG GCT GAT CCT GCA GCA GCA_{21} (the NcoI site is underlined and nucleotides 1 to 21 correspond to the E1^E4 sequence). The reverse primer was TAT ATA ACT CTC GAG T_{279}GG GTG TAG TGT TAC TAT TAC_{258} (the XhoI site is underlined and nucleotides 279 to 258 correspond to the E1^E4 sequence). Recombinant protein expression from pET-28(b)+ constructs are regulated by T7 RNA polymerase which is under the control of the *lac*UV5 promoter, thus IPTG was required to induce expression. In cell culture, WT 16E1^E4 was expressed by transfection of the pMV11.16E1^E4 construct (Davy et al., 2002) and by infection of recombinant adenovirus containing the rAd16E1^E4 construct (Doorbar et al., 2000b). βGAL was expressed by infection of recombinant adenovirus containing the rAdβGAL construct (Fournier et al., 1999). HPV16 E5 was expressed from the vector, pMT3H16E5KC (a kind gift from Dr Dan DiMiao, Department of Genetics, Yale University School of Medicine, CT, USA).

2.5.2 *E.coli* strains used

XL1-Blue supercompetent cells (Stratagene, UK) were used in the mutagenesis protocol and for when new plasmid DNA preps were required. The strain is recombination deficient, endonuclease deficient, and has a mutation in the *hsdR*

endonuclease system so that cleavage of cloned DNA is reduced. BL21 Star™ (DE3) One Shot® cells (Invitrogen, UK) were used to express His-E1^E4 from the pET-28(b)+ vector. The strain was chosen for optimal E1^E4 production because it does not express the Lon or the OmpT proteases and is deficient in its ability to degrade mRNA because of a truncated RNase E enzyme.

2.5.3 Growing *E.coli* cultures

Cells were grown in LB medium containing appropriate antibiotics to select for plasmid maintenance. The antibiotics used were ampicillin (100 μg/ml) for pMV11 and kanamycin (50 μg/ml) for pET-28(b)+. Cultures were incubated at 37 °C, in LB broth with shaking (220 rpm) or LB agar.

2.5.4 Transformation of *E.coli* with DNA

The chemical competent *E.coli* cells, XL1-Blue supercompetent cells or BL21 Star™ (DE3) cells, were used in transformations. One μl of plasmid miniprep or maxiprep DNA (0.1-50 ng of DNA) was added to 50 μl of cells in a pre-chilled 15 ml Falcon 2059 polypropylene tube. The tube was incubated on ice for 30 min then heat-pulsed in a 42 °C water bath for 45 sec. After cooling the tube on ice for 2 min, 1 ml of SOC medium (Invitrogen, UK) was added, then the tube incubated at 37 °C for 1 h with shaking at 220 rpm. The culture (~250 μl) was spread on an LB agar plate (containing 50 μg/ml kanamycin or 100 μg/ml ampicillin) using aseptic technique and the plate incubated overnight at 37 °C. Cultures of any resultant *E. coli* clones were prepared the following day by transferring bacteria from the plate to LB broth using a sterile pipette tip and aseptic technique.

2.5.5 Glycerol stocks

E.coli clones were stored at –80 °C as a 'glycerol stock'. A glycerol stock was prepared by transferring 0.5 ml of a fresh mid-log culture (in LB broth) to a cryogenic vial and adding 0.5 ml of glycerol. This was mixed and immediately stored at –80 °C.

2.5.6 Purifying plasmid DNA

2.5.6.1 Minipreps

Two ml of plasmid-containing *E.*coli cultures (in LB broth with 50 μg/ml kanamycin or 100 μg/ml ampicillin) were grown overnight at 37 °C with shaking at 220 rpm. Plasmid minipreps were prepared using the Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, UK) using the manufacturer's protocol. DNA was eluted using 50 μl of deionised H_2O.

2.5.6.2 Maxipreps

A 5 ml plasmid-containing *E. coli* culture was grown during the day (for ~ 6 h) in LB broth containing 50 μg/ml kanamycin or 100 μg/ml ampicillin (at 37 °C with shaking at 220 rpm). Then, 150-250 ml of LB broth (containing antibiotic) was inoculated with the 5 ml culture, and was incubated overnight at 37 °C with shaking at 220 rpm. The HiSpeed Maxi Kit (Qiagen, UK) was used to produce plasmid DNA maxipreps according to the manufacturer's protocol. DNA was eluted using 1 ml of deionised H_2O.

2.5.7 Quantitation of DNA

DNA concentrations were assayed using a 1201 UV-visible spectrophotometer (Shimadzu, UK). DNA solutions were diluted 1:100-1:500 and their absorbance at 260 nm measured in quartz cuvettes. DNA concentrations were calculated assuming an A_{260} of 1 is equivalent to 50 μg/ml of double-stranded DNA.

2.5.8 TBE agarose gel electrophoresis

To check for the presence or quantity of DNA (for example, mutagenesis PCR products, miniprep DNA and RT-PCR products), the DNA was separated on a 1 % agarose gel, buffered with TBE, containing 1 μg/ml ethidium bromide. Samples were mixed with 10 x loading buffer, loaded into the gel, and electrophoresis was carried out at a constant voltage of 70 V, limiting to 200 mA. DNA bands were visualized by trans-UV illumination and compared to DNA markers (Invitrogen, UK) of known

molecular mass. Digital images were captured with a Kodak Image Station 440 and Digital Science 1D software (Perkin Elmer Life Sciences Ltd., UK).

2.5.9 Site-directed mutagenesis of 16E1^E4

Site-directed mutagenesis was performed using the QuickChange® Site-Directed Mutagenesis kit (Stratagene, UK) according to the manufacturer's protocol. The primers used to make the 16E1^E4 mutants are shown in Table 2.4. They were designed to have the mutation in the middle of the sequence, a melting temperature (T_m) equal to or greater than 78 °C (T_m = 81.5 + 0.41(% GC) – 675/N where N is the number of bases) and a minimum GC content of 40 %. The plasmid in which the mutation was desired was used as the template DNA for a PCR reaction and the relevant pair of primers was added. To make the T54/57A and T51/54/57A mutants, the T57A mutant plasmid was used as the template DNA. The mutagenesis PCR reactions were cycled using Thermal Cycler 200 (GRI, UK) and the programmes used are shown in Table 2.5.

Table 2.4 Primers for mutagenesis

Mutation	Forward primer	Reverse primer
S32A	ccataccaaagccggcgccttgggcac cg	cggtgcccaaggcgccggctttggtat gg
S43A S44A	cgaagaaacacagacgactagccgcc gaccaagatcagagccag	ctggctctgatcttggtcggcggctagt cgtctgtgtttcttcg
S49A	ccagcgaccaagatcaggcccagaca ccggaaacccc	ggggtttccggtgtctgggcctgatctt ggtcgctgg
T51A	caagatcagagccaggcaccggaaac ccctg	caggggtttccggtgcctggctctgat cttg
T54A	ccagacaccggaagcccctgccacac c	ggtgtggcaggggcttccggtgtctgg
T57A	cggaaacccctgccgcaccactaagtt gttg	caacaacttagtggtgcggcaggggtt tccg
T54/57A	ccagacaccggaagcccctgccgcac ca	tggtgcggcaggggcttccggtgtctg g
T51/54/57A	cagagccaggcaccggaagcccctgc cgca	tgcggcaggggcttccggtgcctggc tctg
T57D	gacaccggaaacccctgccgacccact aagttgttgcacag	ctgtgcaacaacttagtgggtcggcag gggtttccggtgtc

Table 2.5 PCR programmes for mutagenesis

pMV11	pET-28(b)+
1) 95 °C, 30 sec	1) 95 °C, 30 sec
2) 95 °C, 30 sec 3) 55 °C, 1 min 4) 68 °C, 4 min	2) 95 °C, 30 sec 3) 55 °C, 1 min 4) 68 °C, 6 min
Repeat steps 2-4 16 times	Repeat steps 2-4 16 times

Following the PCR reaction, the template plasmid DNA was removed by digestion using 10 U of *Dpn* I (part of the kit), leaving only the PCR product. Then XL1-Blue supercompetent cells were transformed by heat shock using 1 µl of this PCR product (see 2.5.4). The cells were incubated on ice for 2 min then mixed with 1 ml of SOC medium and shaken at 220 rpm for 1 h at 37 °C. They were then spread on LB agar plates (containing kanamycin for pET-28(b)+ mutagenesis or ampicillin for pMV11 mutagenesis). The resultant colonies were sequenced to check for correct mutagenesis (see 2.5.10).

2.5.10 Sequencing of 16E1^E4

When 16E1^E4 sequence-containing plasmids were required to be sequenced, minipreps of the DNA were prepared using the Quantum Prep® Plasmid Miniprep kit (Bio-Rad, UK). The sequencing PCR reaction was performed using the Big Dye® Terminator v1.1 Sequencing kit (Applied Biosystems, UK), using 200 ng of the miniprep DNA and 3.2 pmol of primer. Each reaction was performed in duplicate using the forward primer, cttgatcatatggctgatcctgcagcagcaac. The PCR reaction was cycled using Thermal Cycler 200 (GRI, UK). Following the reaction, the DNA was precipitated by adding 2 µl of 3 M sodium acetate (pH 4.6) and 50 µl of ice-cold 95 % ethanol, vortexing briefly, incubating on ice for 10 min and centrifuging at 9000 g for 15 min. The supernatant was carefully removed, the DNA pellet washed with 70 % ethanol and then air-dried for 1 h. Capillary sequencing was performed using MegaBACE™ (GE Healthcare, UK).

2.5.11 Reverse transcription-polymerase chain reaction (RT-PCR) of HPV16 E5

To check for the presence of E5 mRNA in the stably-transfected SiHa cells described in 2.3.7, RT-PCR was performed. The cells were grown to confluency in a 90 mm dish and the total RNA extracted using the RNase-Easy® Mini Kit (Qiagen, UK) and eluted into 50 µl of RNase-free H_2O (part of the kit). One µl of the extracted RNA was added to 25 pmoles of Oligi dT 12-18 mer primer (Invitrogen, UK), 0.5 µl of 10 mM dNTP and ddH_2O, and incubated at 65 °C for 5 min followed by 1 min on ice. Reverse transcription to produce cDNA was then carried out by adding 2 µl of First Strand Buffer (Invitrogen, UK), 0.5 µl of 0.1 M DTT, 0.5 µl of RNasin® (Promega, UK) and 0.5 µl of SuperScript™ III reverse transcriptase (Invitrogen, UK) and incubating at 50 °C for 90 min. The reaction was then inactivated by incubation at 70 °C for 15 min. A 20 µl PCR reaction mixture was made using 2 µl of the cDNA sample, 2 µl of Thermoprime Plus DNA polymerase buffer (Abgene®, UK), 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.5 µl of Thermoprime Plus DNA polymerase (Abgene®, UK) and 10 pmoles of each E5 primer shown below.

forward: CGATGGATCCACCATGACAAATCTTGATACTGCATC
reverse: GCTAGAATTCTTATGTAATTAAAAAGCGTGC

The PCR programme used is outlined below:
Step 1: 94 °C 3 min
Step 2: 94 °C 45 sec
Step 3: 55 °C 45 sec
Step 4: 72 °C 30 sec
Repeat steps 2-4, 29 times.
The PCR reaction was cycled using Thermal Cycler 200 (GRI, UK).

2.6 Protein methods

2.6.1 SDS-PAGE

SDS-PAGE was used to separate proteins according to molecular mass. It was performed using Mini Protean II apparatus (Bio-Rad, UK). 1.5 mm spacers were used and 15 % acrylamide resolving gels were made unless otherwise stated. The

components for preparing 12 % and 15 % resolving gels are shown in Table 2.6. The resolving gel solution was prepared, ensuring that the APS and TEMED were added just before use. This solution was poured into the caster to 3 cm below the top, overlaid with water-saturated butanol and allowed to polymerise for 20 min. Once the resolving gel was set, the butanol was rinsed away with ddH_2O and the stacking gel solution prepared using the components shown in Table 2.6 (the APS and TEMED were added just before use). The stacking gel was poured on top of the resolving gel and a gel comb inserted into the stacking gel solution. After 20 min the comb was removed and the gel positioned in the electrophoresis tank. The tank was filled with SDS electrophoresis buffer and the sample wells rinsed with this buffer.

Table 2.6 Components of the resolving and stacking gels

Stock solutions	10 ml resolving solution	5 ml stacking solution
ddH_2O	3.3 ml (12 %), 2.3 ml (15 %)	3.4 ml
30 % acrylamide	4 ml (12 %), 5 ml (15 %)	0.83 ml
1.5 M Tris pH 8.8	2.5 ml	0 ml
1 M Tris pH 6.8	0 ml	0.63 ml
10 % (w/v) SDS	0.1 ml	0.05 ml
10 % (w/v) APS	0.1 ml	0.05 ml
TEMED	0.004 ml	0.005 ml

Protein samples were then prepared for loading by mixing with an equal volume of 2 x Laemmli's buffer and denaturing by incubating at 95 °C for 2 min. To prepare harvested cell pellets (see 2.3.11) for loading, the pellets were lysed on ice with 2 % SDS in PBS, unless otherwise stated. After lysis, 1 µl of Benzonase® was added to each sample to reduce viscosity caused by DNA and RNA. The cell extract samples were also denatured with Laemmli's buffer as described above. Samples and protein standards (rainbow protein standards (GE Healthcare, UK) or silver stain protein standards (Bio-Rad, UK)) were loaded into the wells of the gel. The gel was then run at a constant voltage of 150 V until the desired separation of the protein-standards was achieved.

2.6.2 Two dimensional (2D) SDS-PAGE

2.6.2.1 Sample preparation

To prepare SiHa cell extracts for 2D SDS-PAGE followed by Western blotting, cells were harvested (see section 2.3.11) and lysed on ice with 2 % SDS in PBS (100 µl per 90 mm dish or 50 µl per 60 mm dish of cells) in the presence of 1 µl Benzonase®. This lysate was diluted 1:3 in ddH_2O (cell extracts that were treated with λ phosphatase (see 2.7.2) were also diluted 1:3 and subsequent steps were identical). This dilution step is to ensure that the SDS concentration is not high enough to interfere with isoelectric focusing (IEF; or in other terms, 'the first dimension'). Thirty µl of the diluted lysate was then mixed with 470 µl of urea-thiourea (U-T) buffer (5 M urea (PlusOne, GE Healthcare, UK), 2 M thiourea (Fluka, Sigma-Aldrich, UK), 2 % CHAPS, 2 % SB 3-10 (Fluka, Sigma-Aldrich, UK), 65 mM DTT (SigmaUltra, Sigma-Aldrich, UK), 20 mM Tris pH 9.5, 0.1 mM EDTA). The sample was spun at 100000 g for 50 min at 16 °C in an TLA-100 ultracentrifuge (Beckman, USA). The supernatant was transferred to a clean 1.5 ml microfuge tube and stored at –80 °C. Prior to using the sample for IEF, the sample was thawed and mixed with immobilised pH gradient (IPG) buffer (GE Healthcare, UK) to make a final IPG buffer concentration of 0.8 % (v/v) and spun in a microcon filter tube (0.22 µm pore; Millipore, UK) at 9000 g for 3 min.

When SiHa cells were prepared for 2D SDS-PAGE followed by silver staining, ten 90 mm dishes were harvested for each IEF run and the cells were lysed directly into 100 µl of U-T buffer. 2 µl of Benzonase® was added and the sample sheared using a 1 ml syringe fitted with a 23 gauge needle. The ultracentrifugation step and all subsequent steps were the same as described above.

2.6.2.2 Performing isoelectric focusing (IEF; the first dimension)

7 cm IPG strips of pH 6-11 or pH 3-10 (GE Healthcare, UK) were rehydrated overnight with 125 µl of U-T buffer in an Immobiline DryStrip Reswelling Tray (GE Healthcare, UK). All strips were overlaid with IPG cover fluid (GE Healthcare, UK) and the tray covered with Saran Wrap® (The Dow Chemical Company, USA).

IEF was performed the following day using the Multiphor II system (GE Healthcare, UK; Figure 2.1). The Immobiline DryStrip tray (GE Healthcare, UK) and DryStrip

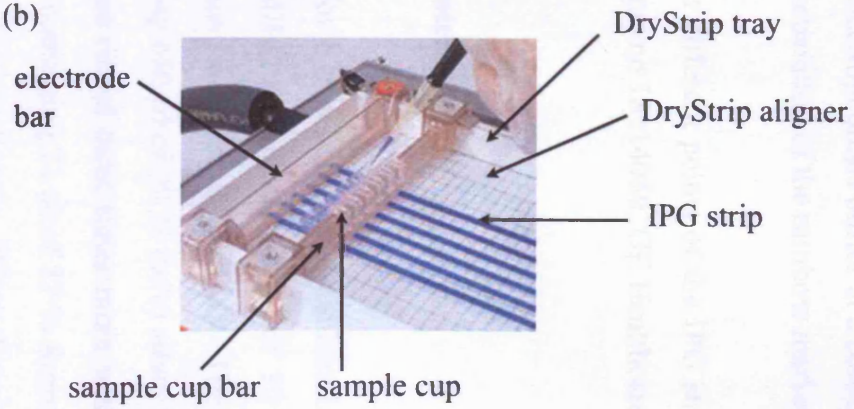
aligner (GE Healthcare, UK) were correctly positioned and then the rehydrated IPG strips were positioned gel-side-up on the aligner. The acidic ends of the strips were near the anode whilst the basic ends were near the cathode. Two electrode strips (GE Healthcare, UK) were moistened with ddH_2O and placed over the acidic and basic ends of the strips, perpendicular to the strips. The anode bar (GE Healthcare, UK) was fitted over the electrode strip at the acidic end and the cathode bar (GE Heatlthcare, UK) was fitted over the electrode strip at the basic end. Sample cups (GE Healthcare, UK) were positioned over the acidic end of the gel using a sample cup bar (GE Healthcare, UK), and the tray was filled with 230 ml cover fluid. One hundred µl of sample (containing a trace of bromophenol blue powder) was applied into each sample cup by pipetting under the surface of the cover fluid. The samples were now ready to run. A MultiTemp III thermostatic circulator (GE Healthcare, UK) was used to set the temperature to 20 °C. An EPS 3501XL power supply (GE Healthcare, UK) was programmed to run seven phases in the order shown in Table 2.7. When the IEF was complete, the second dimension was either performed straight away, or the IPG strips were stored at –80 °C until required.

Table 2.7 IEF programme

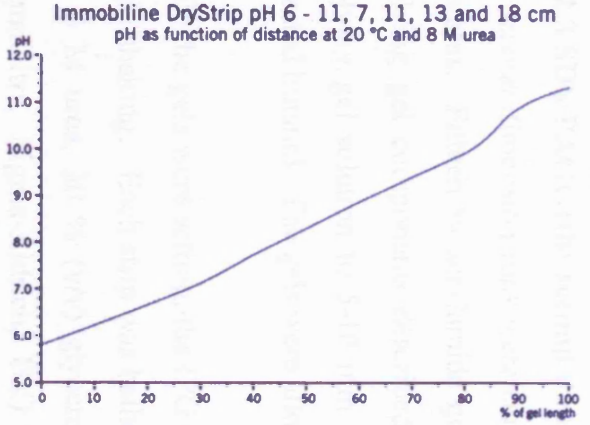
Phase	Voltage (V)	Current (mA)	Resistance (Ohms)	Time (h:min)	Volthours
1	150	2	5	0:30	75
2	300	2	5	2:30	750
3	700	2	5	0:30	350
4	1500	2	5	0:30	750
5	2000	2	5	0:30	1000
6	3000	2	5	0:30	1500
7	3500	2	5	16:00	56000

(a)

(b)



(c)



(d)

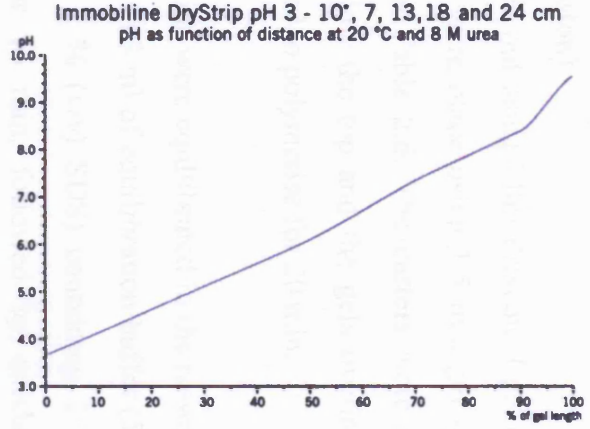


Figure 2.1 IEF apparatus and pH gradient

(a) Image of the Multiphor II IEF system (GE Healthcare). (b) Loading of samples using sample cups. (c and d) Graphs provided by GE Healthcare (cat no 18-1140-60) showing pH as a function of distance along the IPG strip, were used to calculate the pH at which proteins focused in (c) pH6-11 IPG strips and (d) pH3-10 IPG strips.

2.6.2.3 SDS-PAGE (the second dimension)

The second dimension runs were carried out using Mini Protean II gel electrophoresis apparatus. Fifteen % acrylamide gels were made using 1.5 mm gel spacers and the resolving gel components described in Table 2.6. The casters were filled with the resolving gel solution to 5-10 mm below the top and the gels overlaid with water-saturated butanol. The gels were allowed to polymerise for 20 min.

While the gels were setting, the IPG strips were equilibrated in the reswelling tray with gentle shaking. Each strip was bathed in 3 ml of equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (v/v) SDS) containing 2 % (w/v) DTT (SigmaUltra, Sigma-Aldrich, UK) for 15 min, followed by another 15 min in equilibration buffer with 2.5 % (w/v) iodoacetamide (SigmaUltra, Sigma-Aldrich, UK) and a trace of bromophenol blue.

Once the resolving gels were set, the surface of the gels were rinsed with ddH_20. An equilibrated IPG strip was placed on the surface of each gel, avoiding air bubbles between the two gel surfaces. The strips were set in place with 1 % agarose. Rainbow protein standards were loaded by creating a well in the agarose using a strip of 1 mm thick card. Each gel was run in SDS electrophoresis buffer at a constant voltage of 150 V. The gels were run until sufficient separation of the rainbow markers was achieved.

When analysing the results, the pH at different points of the IPG strip was calculated using the charts in Figure 2.1 (catalogue no 18114060, GE Healthcare, UK).

2.6.3 Staining of electrophoresed proteins

2.6.3.1 Silver staining

Gels to be silver stained were fixed for 1 h in 50 % (v/v) methanol. Then they were incubated for 1 min in 50 ml of ddH_2O containing 85 µl of 10 % (w/v) sodium thiosulphate and then rinsed three times for 5 sec with ddH_2O. The gels were stained for 8 min with 50 ml of H_2O containing 650 µl of 20 % (w/v) silver nitrate and 500 µl of 37 % formaldehyde. The gels were rinsed three times more with ddH_2O then the stain developed with 50 ml of ddH_2O containing 21 µl of 37 % formaldehyde, 21 µl of 10 % sodium thiosulphate and 1g of sodium carbonate. When the protein stain became

apparent, the reaction was stopped with 1 % (v/v) acetic acid.

2.6.3.2 Coomassie staining

Gels were stained using the Colloidal Blue Staining kit (Invitrogen, UK). After electrophoresis, gels were fixed for 10 min in 50 % (v/v) methanol and 10 % (v/v) acetic acid. A solution containing Colloidal Coomassie was prepared according to the manufacturer's protocol and gels incubated in this solution overnight. Gels were destained for at least 7 h with deionised H_2O.

2.6.4 Western blotting

Western blotting was performed to detect proteins that have been electrophoretically separated. The protein sample was first run in a denaturing gel (either 1D or 2D SDS-PAGE; see 2.6.1 or 2.6.2 respectively). The separated proteins were then transferred from the gel to a PVDF membrane, Immobilon™ (Millipore, UK). This was achieved using a wet transfer system, Mini Transfer Blot (Bio-Rad, UK; filled with transfer buffer), at a constant voltage of 150 V for 90 min. For detecting active p42/p44 MAPK, a nitrocellulose membrane, Protran® (Schleicher and Schuell Bioscience, Geneflow Ltd., UK), was used instead of Immobilon™.

The membrane was incubated in blocking solution for 1 h at R/T with gentle shaking. For most Western blots, the membrane was blocked in PBS containing 5 % (w/v) Marvel (Premier Brands UK, Ltd., UK) and 0.1 % (v/v) Tween20. When detecting phosphotyrosine, Marvel was replaced with BSA and PBS was replaced with Tris buffered saline (TBS). When detecting active p42/p44MAPK, the membrane was blocked with 2 % (w/v) BSA in TBS.

For most Western blots, the membrane was incubated with primary antibody (see Table 2.8 for a list of antibodies and their working dilutions) for 2 h at R/T with gentle shaking, in PBS, 5 % (w/v) Marvel and 0.1 % (v/v) Tween20. When detecting phosphotyrosine, Marvel was replaced with BSA and PBS was replaced with TBS. When detecting active p42/p44MAPK, the antibody was diluted in TBS containing 0.1 % (w/v) BSA and 0.05 % (v/v) Tween20.

Table 2.8 Primary antibodies used for Western blotting

Target Protein/ Epitope	Primary Antibody	Dilution	Source/ Reference
16E1^E4 epitopes recognised: PWAPKKHR WAPKKHRR APKKHRRL PKKHRRLS KKHRRLSS	TVG402, hybridoma supernatant (mouse monoclonal)	1:10	(Doorbar et al., 1992)
16E1^E4 N-terminus MADPAAATKYPL	Anti-N-terminus (rabbit polyclonal)	1:800	(Doorbar et al., 1997)
Phosphotyrosine	anti- phosphotyrosine, clone PY20 (mouse monoclonal)	1:300	Neomarkers, UK
Keratin	anti-keratin 18/8, 8592 (rabbit polyclonal)	1:1000	Dr Bishr Omary (Stanford University, USA)
Active p42/p44MAPK	anti-ACTIVE® MAPK (rabbit polyclonal)	1:5000	Promega, UK
GAPDH	anti-GAPDH, MAB374 (mouse monoclonal)	1:800	Chemicon Europe Ltd., UK

For most Western blots, the membrane was then washed in PBS with 0.1 % (v/v) Tween20 three times at 15 min intervals. When detecting phosphotyrosine, the PBS was replaced with TBS. When detecting active p42/p44MAPK, the membrane was washed in TBS with 0.05 % (v/v) Tween20.

For most Western blots, the membrane was then incubated for 1 h at R/T with gentle shaking in PBS with 5 % (w/v) Marvel and 0.1 % (v/v) Tween20, containing HRP-conjugated anti-mouse or HRP-conjugated anti-rabbit IgG (GE Healthcare, UK) diluted to 1:3000. When detecting phosphotyrosine, Marvel was replaced with BSA and PBS was replaced with TBS. When detecting active p42/p44MAPK, the anti-rabbit antibody was diluted in TBS containing 0.1 % (w/v) BSA and 0.05 % (v/v) Tween20. Following this, the membranes were washed three times as described above.

Binding of HRP-conjugated antibody was visualized by Enhanced Chemiluminescence (ECL; GE Healthcare, UK), with blots being exposed to Kodak MXB X-ray film (X-ograph imaging systems, UK). Films were developed using a Fujifilm FPM-3800A processor (UK).

2.6.5 Expression, purification and refolding of His-E1^E4

C-terminus hexa-histidine tagged E1^E4 with a 'leucine, glutamic acid' linker (His-E1^E4) was expressed from a pET-28(b)+ construct (Novagen, UK) in BL21 Star™ cells (Invitrogen, UK). The cloning of this construct and optimisation of the following expression and purification steps, was done by Dr Pauline McIntosh (NIMR). The glycerol stock of this construct (in BL21 Star™ (DE3)) was streaked onto an LB agar plate (containing 50 μg/ml kanamycin) and the plate incubated overnight at 37 °C. A colony was scraped and transferred to 10 ml of LB broth (containing kanamycin) and this culture grown overnight at 37 °C with shaking. The following morning, the culture was diluted in 1000 ml of LB broth (containing kanamycin) and grown at 37 °C with shaking. When the culture had an OD of 0.6 at wavelength 600 nm, the bacteria were induced to express His-E1^E4 by addition of 0.2 mM IPTG and the temperature reduced to 30 °C. Three h after induction, the culture was centrifuged at 3000 g for 15 min at 4 °C to pellet the bacteria. The pellet was resuspended in 100 ml of pH 7 urea buffer (8 M urea, 0.01 M Tris, 0.1 M NaH_2PO_4) containing 25 mM Complete Protease Inhibitor. The resuspension was sonicated (at 34 Watts, with a 15 sec pulse followed by a 15 sec pause repeated sixteen times) by a Branson Digital Sonifier®, then centrifuged at 15000 g for 30 min at 4 °C. The supernatant was clarified bacterial lysate. One ml of Talon Metal Affinity Resin (BD Biosciences, UK) was added to an Econo-Pac column (Bio-Rad, UK) and equilibrated by washing with 10 ml of the pH 7 urea buffer. The lysate was added to the column and allowed to flow through. The column was washed five times with 10 ml of the pH 7 urea buffer. The His-E1^E4 was eluted into five 1 ml aliquots using a pH 4.3 urea buffer (8 M urea, 0.01 M Tris, 0.1 M NaH_2PO_4). The third 1 ml elution was generally the purest and the most concentrated sample, and was used in experiments. The protein samples were refolded for experiments (unless otherwise stated) by dialysing into PBS (containing 2 mM DTT) overnight at 4 °C using Slide-A-Lyzer Mini Dialysis Units with a 5000 Da cut-off (Pierce, UK).

2.6.6 Quantitation of protein

2.6.6.1 Quantitation by spectrometry

Protein concentrations were assayed against a blank using a 1201 UV-visible spectrophotometer (Shimadzu, UK). Protein concentrations were calculated using the formula below. The extinction coefficient of WT His-E1^E4 and His-E1^E4 with point mutations is 18470 (calculated by the ProtParam Tool, ExPASy, http://ca.expasy.org/cgi-bin/protparam). The molecular mass (also calculated by ExPASy) of WT His-E1^E4 and His-E1^E4 with point mutations, is 11, 100 Da (to the nearest 0.1 k Da.

$$\mu g/\mu l = \left[\frac{OD_{280} - OD_{320}}{\text{Extinction Coefficient}} \right] \text{ X mass in daltons}$$

2.6.6.2 Quantitation with a protein assay

When a protein assay was performed, the Bio-Rad Protein Assay Dye Reagent Concentrate was used and the manufacturer's protocol followed. BSA standards ranging from 0.1 to 1.0 mg/ml were used to make a standard curve.

2.6.7 Mass spectrometry (MS)

2.6.7.1 Sample preparation

1D or 2D SDS-PAGE was performed (see 2.6.1 or 2.6.2 respectively) using the protein sample to be analysed by MS. The gels were stained using Colloidal Blue (Invitrogen, UK) as described in the manufacturer's protocol. The gels were destained overnight in deionised water. The following sample preparation steps were conducted in a sterile tissue culture hood (as keratin contamination is a common problem with MS).

The stained bands of interest were excised, cut into 2 mm x 1 mm pieces and placed in 0.5 ml non-coloured low adhesion siliconised tubes (Eppendorf, UK) (that have been pre-rinsed with Buffer A (200 mM ammonium bicarbonate (Merck, UK), 50 % (v/v) acetonitrile (Rathburn Chemicals Ltd, Scotland, UK)). The SDS and Coomassie were extracted from the gel pieces by 30 min incubations in 500 µl of Buffer A until the

Coomassie stain was no longer apparent. The protein was reduced by incubating the gel pieces in 200 µl Buffer A containing 20 mM DTT (SigmaUltra, Sigma-Aldrich, UK) for 1 h. The DTT was then removed by washing with Buffer A and the cysteine residues alkylated by incubating the sample with 200 µl of Buffer A containing 5 mM iodoacetamide (SigmaUltra, Sigma-Aldrich, UK) for 20 min (in the dark). The gel pieces were then rinsed twice with 500 µl of Buffer B (20 mM ammonium bicarbonate, 50 % acetonitrile) and 500 µl of neat acetonitrile added for 15 min. Following this, all liquid was removed by pipetting and the gel pieces left to air-dry for 1 h. The sample was then stored at –20 ° C.

Before performing MS, each gel piece was incubated overnight (in a 32 ° C oven) in a small volume (just enough to fully immerse the gel piece) of 2 µg/ml sequencing grade chymotrypsin (Roche, UK) in 5 mM ammonium bicarbonate. The MS steps described below were performed by Dr Steve Howell (NIMR). Results were analysed by Dr Steve Howell using software obtained as part of the mass spectrometer package.

2.6.7.2 MALDI MS

The digested protein was acidified with 1/10 volume of 2 % trifluoroacetic acid. A Reflex II MALDI TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a nitrogen laser and a Scout-384 probe was used for MS. Thin layer matrix surfaces of α-cyano-4-hydroxycinnamic acid mixed with nitrocellulose were prepared. A small volume (0.4 µl) of the acidified supernatant was deposited onto the thin layer and allowed to dry prior to rinsing with water. An accelerating voltage of 26 kV was used with detector bias gating set to 2 kV.

2.6.7.3 Nanospray MS

The digested protein was acidified with 1/10 volume of 2 % trifluoroacetic acid. The supernatant was loaded onto a 2 mm x 0.8 mm C18 microcolumn (LC Packings, Amsterdam, Netherlands), washed and step eluted with 60 % methanol, 0.1 % formic acid directly into an Econo 12 nanospray needle (New Objective Inc, Cambridge MA). Nanospray mass spectra were acquired on an LCQ Quadruple ion trap mass spectrometer (ThermoQuest Corporation, Austin, TX) equipped with a nanospray source (Protana, Odense, Denmark) operated at a spray voltage of 800 V and a capillary temperature of 150 °C. For collision-induced fragmentation, mass spectra

were acquired at a collision energy of 30 % and a parent ion isolation width of 3 Da.

2.7 Other molecular biology and biochemistry methods

2.7.1 *In Vitro* kinase assays

Radioactive i*n vitro* kinase assays were carried out using protein substrates, γ-^{32}P-ATP and recombinant kinases. All kinases used were commercially available and these are listed in Table 2.9, accompanied by the 10 x reaction buffer components. His-E1^E4, myelin basic protein, α-casein or GSK-3 fusion protein (Cell Signalling Technology, UK) were used as substrates. The reaction mixture consisted of 1 µg of substrate, 2.5 µl of 10 x reaction buffer, 2.5 µl of 10 mM ^{32}P-ATP (100-500 µCi/µmol; GE Healthcare, UK), 1 µl of kinase (CKII, CDK1, CDK2, p42MAPK, PKA, PKB or PKCα) and ddH_2O to make the total volume 25 µl. CaMKII was activated by autophosphorylation prior to substrate phosphorylation, by incubating 1 µl of the kinase for 10 min at 30 °C in a 20 µl reaction containing 2.5 µl of 10 x reaction buffer, 2 mM $CaCl_2$, 1.2 µM calmodulin, 100 µM ATP (non-radioactive) and ddH_2O. One µg of substrate, 10 mM γ-^{32}P-ATP (100-500 µCi/µmol) and ddH_2O were then added to the mixture to make the total volume 25 µl. Substrate and kinase reaction mixtures were incubated for 30 min at 30 °C unless otherwise stated, and the reactions stopped by addition of 25 µl of 2 x Laemmli's buffer and heating at 95 °C for 3 min.

SDS-PAGE was carried out on the samples ensuring that the dye front was run-off (to remove free ATP). The acrylamide gel was then fixed for 20 min in 40 % (v/v) methanol, 10 % (v/v) acetic acid and 1 % (v/v) glycerol. The gel was dried, exposed to a light-blanked PhosphorImager plate overnight and then analysed using a Storm860 PhosphorImager (GE Healthcare, UK) and ImageQuant 5.0 software.

Table 2.9 Protein kinases used and their reaction buffer components

kinase	10 X reaction buffer
CaMKII (New England Biolabs, UK)	0.5 M Tris, 0.1 M $MgCl_2$, 20 mM DTT, 1 mM Na_2 EDTA, pH 7.5
CDK1/cyclin B (New England Biolabs, UK)	0.5 M Tris, 0.1 M $MgCl_2$, 10 mM EGTA, 20 mM DTT, 0.1 % (v/v) Brij 35, pH 7.5
CDK2/cyclin A (New England Biolabs, UK)	0.5 M Tris, 0.1 M MgCl2, 10 mM EGTA, 20 mM DTT, 0.1 % Brij 35, pH 7.5
CKII (New England Biolabs, UK)	0.2 M Tris, 0.5 M KCl, 0.1 M $MgCl_2$, pH 7.5
$_P$42MAPK (New England Biolabs, UK)	0.5 M Tris, 0.1 M $MgCl_2$, 10 mM EGTA, 20 mM DTT, 0.1 % (v/v) Brij 35, pH 7.5
PKA (Promega, UK)	0.4 M Tris, 0.2 M magnesium acetate, pH 7.4
PKB (Cell Signaling Tecnology, UK)	0.25 M Tris, 50 mM β-glycerolphosphate, 20 mM DTT, 1 mM Na_3VO_4, 0.1 M $MgCl_2$, pH 7.5
PKCα (Calbiochem, UK)	100 mM HEPES, 1 M $MgCl_2$, $CaCl_2$, 50 mM DTT, 10 mM EGTA, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) phosphatidylserine (Avanti® Polar Lipids, Inc., USA), 0.1 % (w/v) diacylglycerol (Avanti® Polar Lipids, Inc., USA), pH 7.4

When protein phosphorylation was required for MS, kinase reactions were carried out as described above but non-radioactive ATP was used.

2.7.2 lambda (λ) phosphatase experiments

A 90 mm plate of rAdE1^E4-infected SiHa cells was harvested and the cells lysed in 50 µl of 0.8 % (v/v) Empigen. Twenty µl of the lysate was incubated with 1 x λ phosphatase buffer (New England Biolabs, UK), 2 mM $MnCl_2$ (New England Biolabs, UK) and 1 µl of λ phosphatase (New England Biolabs, UK) for 30 min at 30 °C to allow protein dephosphorylation. A λ phosphatase-negative sample was always included, where the phosphatase was replaced by 1 µl of ddH_2O.

2.7.3 Immunoprecipitation of 16E1^E4

Three 90 mm plates of rAdE1^E4-infected SiHa cells were harvested 24 h post-infection. The cells were resuspended in 500 µl of 0.8 % (v/v) Empigen in PBS then incubated on ice for 30 min. The immunoprecipitation in this study was carried out for detection of potential phosphotyrosine, so the cells were lysed in the presence of 1mM sodium vanadate, an inhibitor of tyrosine phosphatases (Lau et al., 1989). The lysate was centrifuged for 5 min at 9000 g, then 50µl of the supernatant transferred to a clean 1.5 ml microfuge tube. Nine hundred and forty µl of PBS (containing 1 % (v/v) NP40, 100 µg/ml BSA and 25 mM Complete Protease Inhibitor) and 10 µl of anti-MBP16E1^E4 rabbit polyclonal IgG (Doorbar et al., 1997) was added and the mixture rotated at 4 °C for 1 h. A 50 µl slurry (50 % (v/v)) of Protein G sepharose beads (GE Healthcare, UK; pre-washed with PBS to remove ethanol) was then added and the mixture rotated for a further 1 h. The 1.5 ml tube was centrifuged at 1000 g for 1 min, the supernatant removed and the beads washed three times with PBS (by centrifuging at 1000 g for 30 sec and carefully pipetting the liquid out with a glass pipette). The beads were then resuspended in 50 µl of 2 x Laemmli's buffer and incubated at 96 °C for 5 min. The sample was then analysed by SDS-PAGE and Western blotting.

2.7.4 Immunoprecipitation of keratin followed by incubation with His-E1^E4

SiHa cells (3 X 10^6 cells) were harvested and the pellet resuspended in 500 µl of Empigen buffer (1 % Empigen (v/v) and 25 mM Complete Protease Inhibitor in PBS). This was incubated on ice for 30 min and then centrifuged at 9000 g for 10 min. Fifty µl of the supernatant was added to 150 µl of Empigen buffer and 2 µl of the anti-keratin 8/18 mouse monoclonal, L2A1 (a kind gift from Dr Bishr Omary, Palo Alto VA Medical Center and Stanford University School of Medicine, CA, USA). The sample was mixed and incubated on ice for 1 h. For negative controls, the cell extract (keratin) or L2A1 were omitted. A 50 µl slurry (50 % (v/v)) of Protein G sepharose beads (GE Healthcare, UK; pre-washed with PBS to remove ethanol) was then added and the mixture rotated at 4 °C for 1 h. The beads were then washed three times (by centrifuging at 1000 g for 30 sec and carefully pipetting the liquid out with a glass pipette) with 400 µl of Empigen buffer and then once with 400 µl of NP40 buffer (0.5 % (v/v) NP40, 0.125 % (w/v) gelatin, 25 mM Complete Protease Inhibitor, in PBS). Four hundred µl of NP40 buffer containing 0.4 µg of refolded His-E1^E4 (see section 2.6.5) was added to the beads and the sample rotated at 4 °C for 2 h. The beads were then washed four times with NP40 buffer and resuspended in 25 µl of 2 x Laemmli's buffer. The sample was heated for 5 min at 96 °C, centrifuged at 1000 g for 1 min, and analysed by Western blotting.

2.7.5 Solubility fractionation

SiHa cells were grown in 60 mm Nunc dishes, transfected and harvested 24 h post-transfection (unless otherwise stated). The pellets were resuspended in 80 µl/dish of NP40 lysis buffer (0.5 % (v/v) NP40 in PBS with 25 mM Complete Protease Inhibitor). Each sample was then rotated at 4 °C for 30 min. The sample was subsequently pelleted by centrifugation (10000 g, 10 min, 4 °C). The supernatant was transferred to a 1.5 ml microcentrifuge tube and stored on ice. This was called the 'NP40-soluble fraction'. The resultant pellet was resuspended in 80 µl/dish of 0.8 % (v/v) Empigen in PBS with 25 mM complete Protease Inhibitor cocktail, using a 1 ml syringe fitted with a 23 gauge needle, and then rotated at 4 °C for 30 min. The extract was centrifuged and the supernatant stored on ice and this was called the 'Empigen-soluble fraction'. The remaining pellet was resuspended in 80 µl/plate of 9 M urea in PBS and

subsequently stored on ice, and this was termed the 'insoluble fraction' or the 'urea-soluble fraction'. The fractions were analysed by SDS-PAGE and Western blotting.

2.7.6 Antibody production

Phosphorylated and unphosphorylated 16E1^E4 peptides were produced, conjugated to a carrier protein and injected into rabbits in order to produce polyclonal phosphospecific antibodies.

2.7.6.1 Peptide conjugation to carrier protein

Peptide sequences of 16E1^E4 (10-13 amino acids long) containing the target amino acid (phosphorylated or unphosphorylated) in the middle of the sequence were synthesised by Peter Fletcher (NIMR). The FastMoc solid phase peptide synthesis method was used, the peptides purified using reverse-phase high performance liquid chromatography (HPLC) and then freeze-dried (Tiburu et al., 2003). A cysteine group was included at the end of each sequence to enable conjugation with a maleimide-containing carrier protein via the cysteine's free sulfhydryl (-SH) group (Figure 2.2). The carrier protein used was mariculture keyhole limpet haemocyanin (mcKLH) pre-activated to contain maleimide groups, and the conjugation was performed using the Imject® Maleimide Activated mcKLH Kit (Pierce, UK). KLH was used because it induces a strong antibody response due to its large mass (> 106 kDa) and it has the advantage of being a non-mammalian protein. The manufacturer's protocol was followed; 2 mg of peptide was dissolved in 300 µl of the kit's conjugation buffer and allowed to mix with the pre-activated KLH for 2 h at R/T. Cysteine assays were performed to ensure that conjugation was efficient (see 2.6.6.2).

After conjugation, the peptide-KLH sample (the antigen) was dialysed into PBS by loading the sample into a Slide-A-Lyzer® cassette with a MW cut off of 10,000 Da (Pierce, UK) and then immersing the cassette into PBS, stirring overnight at 4 °C. After dialysis the protein concentration was measured using a protein assay (see 2.6.6.2). A concentration of 0.1 – 0.2 mg/ml of antigen was used in this study. The antigen was filter-sterilised through a 0.2 µM filter and twelve 0.5 ml aliquots prepared for sending to Harlan Sera-Lab (Loughbourgh, UK).

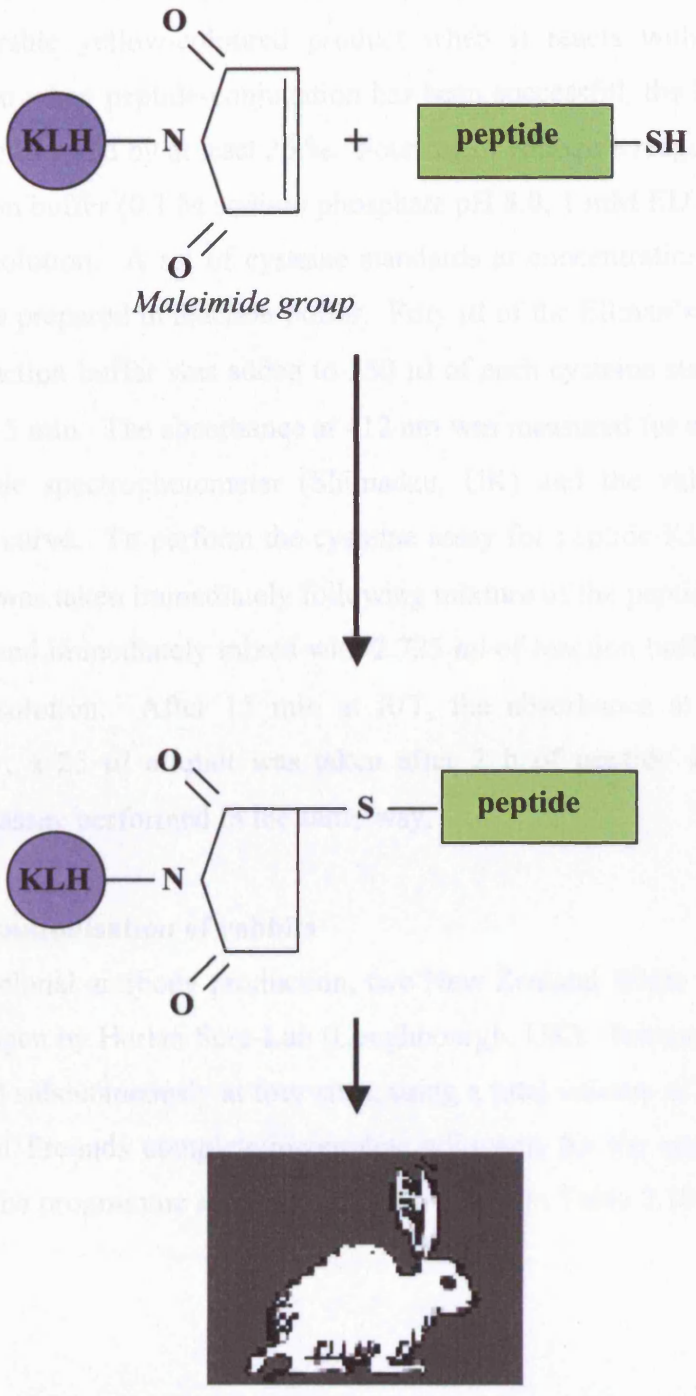


Figure 2.2 Outline of peptide-carrier protein coupling
mcKLH, pre-activated to contain maleimide groups, was coupled to a cysteine-containing peptide. The complex was then used to immunise rabbits to produce an antibody response against the peptide.

2.7.6.2 Cysteine assay

Cysteine assays were performed using Ellman's reagent (Pierce, UK), also known as DTNB (5,5' –dithio-bis-(2-nitrobenzoic acid). A solution of Ellman's reagent produces a measurable yellow-coloured product when it reacts with free –SH (sulfhydryl) groups, so when peptide-conjugation has been successful, the level of free –SH groups should be lowered by at least 25 %. Four mg of Ellman's reagent was dissolved in 1 ml of reaction buffer (0.1 M sodium phosphate pH 8.0, 1 mM EDTA) to produce Ellman's reagent solution. A set of cysteine standards at concentrations ranging from 0 to 1.5 mM were prepared in reaction buffer. Fifty µl of the Ellman's reagent solution and 2.5 ml of reaction buffer was added to 250 µl of each cysteine standard, mixed and left at R/T for 15 min. The absorbance at 412 nm was measured for each sample using a 1201 UV-visible spectrophotometer (Shimadzu, UK) and the values plotted to obtain a standard curve. To perform the cysteine assay for peptide-KLH conjugation, 25 µl of solution was taken immediately following mixture of the peptide with the KLH (section 2.6.6.1) and immediately mixed with 2.725 ml of reaction buffer and 50 µl of Ellman's reagent solution. After 15 min at R/T, the absorbance at 412 nm was recorded. Similarly, a 25 µl aliquot was taken after 2 h of peptide and KLH mixing, and a cysteine assay performed in the same way.

2.7.6.3 Immunisation of rabbits

For polyclonal antibody production, two New Zealand White rabbits were injected for each antigen by Harlan Sera-Lab (Loughbourgh, UK). Immunisations and boosts were delivered subcutaneously at four sites, using a total volume of 1 ml (0.5 ml antigen and 0.5 ml of Freunds complete/incomplete adjuvant) for the immunisation and for each boost. The programme followed is shown below in Table 2.10.

Table 2.10 Immunisation programme for polyclonal antibody production in rabbits

Day No.	Action
0	Pre-bleed + Immunisation (Freunds Complete Adjuvant)
14	Boost 1 (Freunds Incomplete Adjuvant)
28	Boost 2 (Freunds Incomplete Adjuvant)
35	Test bleed 1
42	Boost 3 (Freunds Incomplete Adjuvant)
49	Test bleed 2
56	Boost 4 (Freunds Incomplete Adjuvant)
63	Test bleed
70	Boost 5 (Freunds Incomplete Adjuvant)
77	Terminal bleed

2.8 Statistical analysis

Means and standard deviations were calculated using Microsoft Excel. Where bar graphs were used to display the data, the mean values were shown and error bars used to represent the standard deviation. The unpaired *T*-test with Welch's correction was performed using GraphPad InStat version 3.06 (Welch's correction does not assume equal standard deviations).

Chapter 3: Analysis of 16E1^E4 *In Vitro* Phosphorylation Sites

3.1 Introduction

A phosphorylated form of 16E1^E4 has been reported but not characterised in any detail (Wang et al., 2004). To initiate a study on the phosphorylation of 16E1^E4, the first aim was to map the phosphorylation site(s). 16E1^E4 was phosphorylated *in vitro* and then the phosphorylation site(s) investigated using mass spectrometry (MS). This approach for phosphorylation studies was also used for 11E1^E4 (Bryan et al., 2000). The E1^E4 used was bacterially-expressed histidine-tagged 16E1^E4 (His-E1^E4) as this was easy to produce and purify. The kinases used were recombinant, commercially available protein kinases, selected based on the presence of their consensus sequence within 16E1^E4 (further discussed in this chapter). Some of these kinases have previously been reported to phosphorylate other E1^E4 proteins, so it was important to test for their ability to phosphorylate 16E1^E4. MS was used as the method for phosphorylation site mapping, for its ease, safety and ability to provide results with low protein levels, as compared to other mapping techniques discussed in 3.1.1.1.

3.1.1 Phosphorylation site mapping

3.1.1.1 Traditional techniques

Traditional techniques for phosphoprotein analysis involve incorporating ^{32}P into cellular proteins by incubating cells with radiolabelled phosphorus (Haystead and Garrison, 1999; Van der Geer et al., 1999). The phosphoproteins are then detected (e.g. by isoelectric focusing (IEF) or high-performance liquid chromatography (HPLC)). The phosphoamino acid content can be determined by complete hydrolysis of the protein into amino acids and two-dimensional thin-layer chromatography, a technique allowing separation of phospho-serine, -threonine and -tyrosine. The site(s) of phosphorylation can be determined by proteolytic digestion of the radiolabeled protein, separation of the peptides (e.g. by two-dimensional thin-layer chromatography) followed by peptide sequencing using Edman degradation.

3.1.1.2 Mass spectrometry (MS)

The traditional methods of phosphorylation mapping require significant levels of radioactive protein, therefore in recent years they have been superseded by MS techniques (Mann et al., 2001; Mann et al., 2002; McLachlin and Chait, 2001). The basic components of MS were pioneered early in the last century by JJ Thompson (Thompson, 1913). MS instruments can ionise molecules in the 'ionisation source' and then sort them according to mass/charge (m/z) ratio in the 'analyzer' to produce a spectrum displaying m/z in the x-axis and relative intensity in 'arbitrary units' in the y-axis (Figure 3.1). In many cases the charge is equal to +1, so the m/z value is equal to the molecular mass in Daltons. Addition of a phosphate group will increase the m/z value by 80 units (HPO_3^- = 80 Da). The relative intensity is often a loose correlation with the actual relative amount as it may depend on how easily a molecule is ionised and not just on its abundance. Phosphopeptides are not easily ionisable so their peaks are often weak in intensity even if they are present at high levels.

For mapping phosphorylation sites of 16E1^E4, in-gel proteolyitic digestion of the protein was followed by analysis of the resultant peptides using matrix-assisted laser desorption/ionisation time-of-flight (MALDI TOF) MS or nanospray quadrupole ion trap MS. In MALDI, the sample to be analysed is dissolved in a matrix and allowed to crystallise (Karas and Hillenkamp, 1988). Laser pulses are then used to generate gaseous ions for MS. TOF refers to the system used to analyse the ions (Wiley and McLaren, 1955). With TOF, the ions are accelerated to a fixed amount of kinetic energy so smaller ions have a higher velocity (or a lower time-of-flight) and are detected before larger ions. Nanospray MS generates ions by allowing the liquid sample to flow through a needle at high voltage and electrostatically disperse or 'electrospray' (Wilm and Mann, 1996). The quadrupole ion trap is a different type of analyser to TOF. Ions are trapped between electrodes by use of radio frequency electric fields. Ions with increasing m/z can be sequentially ejected from the trap by varying the applied voltage, thus allowing a spectrum to be generated. Data from MALDI and from nanospray MS can therefore allow identification of phosphopeptides.

Nanospray quadrupole ion trap MS coupled with collision-induced fragmentation can assist in more precise mapping of phosphorylation sites (Marina et al., 1999; Ogueta et al., 2000). With this technique, any ion suspected to be a phosphopeptide can be

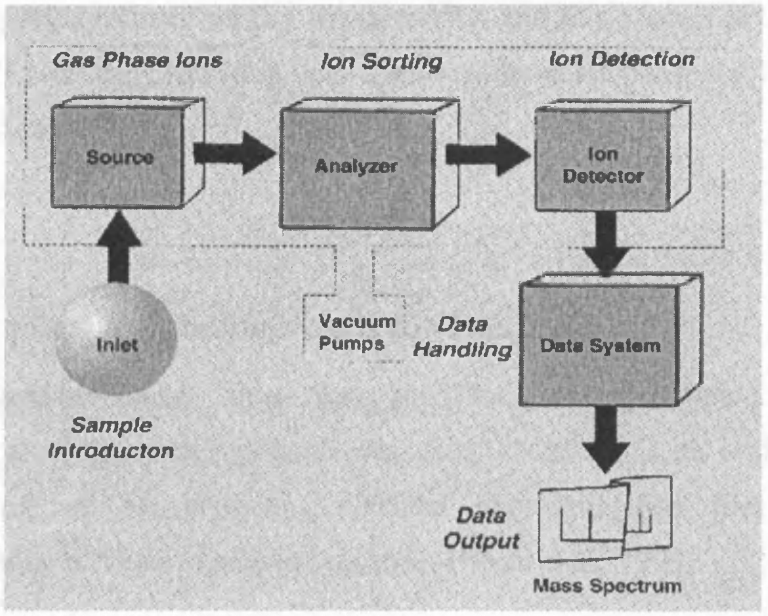


Figure 3.1 Outline of mass spectrometry
The basic functional components of a mass spectrometer is shown in this block diagram (www.asms.org/whatisms/p4.html). The sample enters the vacuum chamber and is transformed into the gas phase and ionised. The mass analyser sorts the gas phase ions according to their mass/charge ratio (m/z). The ions are collected by a detector where they are converted into an electrical current. The data output shows the magnitude of the electrical signals as a function of m/z and this is the mass spectrum.

isolated within the ion trap. The ion can be bombarded with atoms of an inert gas (e.g. helium) causing dissociation of the ion to produce smaller ions. This fragmentation step can be described as 'MS^2'. An ion produced by MS^2 can also be selected for the ion trap and subjected to even further fragmentation and this process is described as MS^3. MS^2 and if necessary, MS^3, can help to narrow down the phosphorylation site or even identify the exact residue.

3.2 Predicted phosphorylation events for 16E1^E4

To predict phosphorylation sites for 16E1^E4, the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) was used. NetPhos is an artificial neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences (Blom et al., 1999).

Crystallisation studies have suggested that ~7-12 residues surrounding the phosphate acceptor are in contact with the kinase active site (Songyang et al., 1994), so NetPhos was designed to search for common patterns in the surrounding residues based on studies of a large set of verified phosphorylation sites. Examples of common features include the fact that tryptophan and cysteine rarely surround phosphorylated serines and basic residues are commonly found at positions –2 and –3 of phosphorylated threonines. Using these patterns, NetPhos predicts a score (i.e. probability) of each serine, threonine and tyrosine in a protein sequence, being phosphorylated. For 16E1^E4, the amino acids with a score above 0.5 were, threonine 21, serine 32, serine 43, serine 44, serine 49 and threonine 51, with serines 32, 43 and 44 having scores above 0.8 (Figure 3.2a).

Another prediction tool used was ScanProsite (http://ca.expasy.org/tools/scanprosite). ScanProsite scans the query protein sequence against the Prosite database (Falquet et al., 2002) of protein families and domains, which includes the phosphorylation site consensus for a few well-characterised kinases; PKA and cGMP-dependent protein kinases (PKG), tyrosine kinase, receptor tyrosine kinase and CKII. For 16E1^E4, the phosphorylation consensus sites recognized by ScanProsite, were for PKA at serine 43

and for CKII at serine 44 (Figure 3.2b). These kinases were therefore included in the list of kinases to test for the ability to phosphorylate 16E1^E4 *in vitro*.

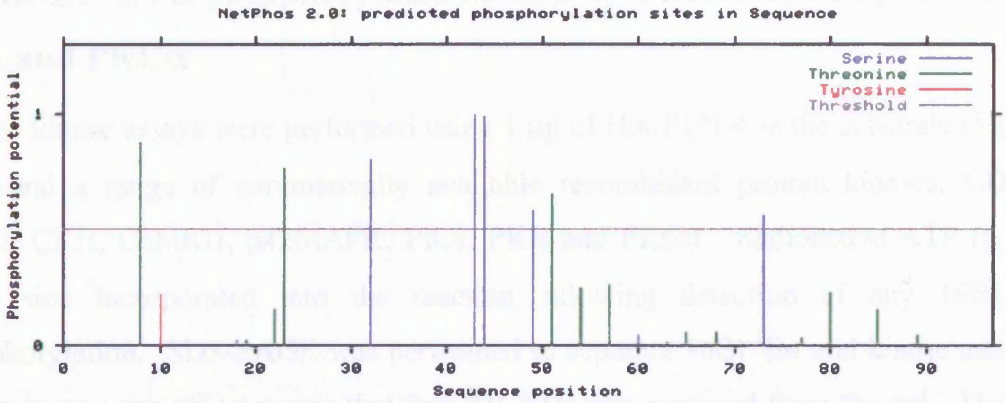
To decide which other protein kinases to test, the high NetPhos-scoring (>0.5) residues were analysed to see which (if any) protein kinase consensus sites they fit (Figure 3.3). It was recognized that threonine 23 falls in both the CDK consensus site (Moreno and Nurse, 1990; Stevenson-Lindert et al., 2003) and a MAPK consensus site (Gonzalez et al., 1991), serine 32 falls in a PKC consensus site (Pearson and Kemp, 1991) and a MAPK consensus site, serine 43 falls in the PKA (Kennelly and Krebs, 1991; Zetterqvist et al., 1976), PKC and calmodulin kinase II (CaMKII; Payne et al., 1983) consensus sites, serine 44 falls in the CaMKII, PKC and CKII (Russo et al., 1992) consensus sites and threonine 51 falls in the MAPK consensus site. PKA has been previously been shown to phosphorylate 1E1^E4 (Grand et al., 1989) and both PKA and p42MAPK have been reported to phosphorylate 11E1^E4 (Bryan et al., 2000).

Importantly, several of these kinases are involved in cell proliferation, growth and differentiation of keratinocytes in the epithelium, for example, MAPK (Mitev et al., 1995; Seo et al., 2004), CaMKII (Praskova et al., 2002) and in particular, PKCα (Bollag et al., 1993; Tibudan et al., 2002; Yang et al., 2003). PKB (also known as Akt) is also involved in keratinocyte survival and differentiation (Calautti et al., 2005; Janes et al., 2004) and although its consensus sequence, R-X-R-X-X-S/T (Alessi et al., 1996), is not present in 16E1^E4, it was also tested for its ability to phosphorylate His-E1^E4.

Figure 3.2 NetPhos and ScanProsite phosphorylation predictions for 16E1^E4

(a) Phosphorylation predictions by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) for each serine (blue), threonine (green) and tyrosine (red) are shown graphically and in a tabular form. The predictions are given as a probablility (or maximum score of 1). The threshold used by NetPhos for describing a high probability is a score above 0.5. Residues with scores above 0.5 are serines 32, 43, 44, 49 and 73 and threonines 8, 23 and 51. (b) ScanProsite (http://ca.expasy.org/tools/scanprosite) was used to scan 16E1^E4 against the Prosite database. This recognised a PKA phosphorylation consensus site at residues 40-43 and a CKII phosphorylation consensus site at residues 44-47.

(a)



1 46

MADPAAATKYPLLKLLGSTWPTTPPRPIPKPSPWAPKKHRRLSSDQ

47 92

DQSQTPETPATPLSCCTETQWTVLQSSLHLTAHTKDGLTVIVTLHP

Serine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	18	KLLGSTWPT	0.016	.
Sequence	32	IPKPSPWAP	0.806	*S*
Sequence	43	HRRLSSDQD	0.995	*S*
Sequence	44	RRLSSDQDQ	0.990	*S*
Sequence	49	DQDQSQTPE	0.582	*S*
Sequence	60	ATPLSCCTE	0.041	.
Sequence	72	TVLQSSLHL	0.003	.
Sequence	73	VLQSSLHLT	0.566	*S*
		^		

Threonine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	8	PAAATKYPL	0.875	*T*
Sequence	19	LLGSTWPTT	0.017	.
Sequence	22	STWPTTPPR	0.155	.
Sequence	23	TWPTTPPRP	0.768	*T*
Sequence	51	DQSQTPETP	0.655	*T*
Sequence	54	QTPETPATP	0.248	.
Sequence	57	ETPATPLSC	0.265	.
Sequence	63	LSCCTETQW	0.013	.
Sequence	65	CCTETQWTV	0.054	.
Sequence	68	ETQWTVLQS	0.053	.
Sequence	77	SLHLTAHTK	0.033	.
Sequence	80	LTAHTKDGL	0.228	.
Sequence	85	KDGLTVIVT	0.156	.
Sequence	89	TVIVTLHP-	0.042	.
		^		

Tyrosine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	10	AATKYPLLK	0.218	.
		^		

(b)

1 47

MADPAAATKYPLLKLLGSTWPTTPPRPIPKPSPWAPKKHRRLSSDQD

PKA CKII

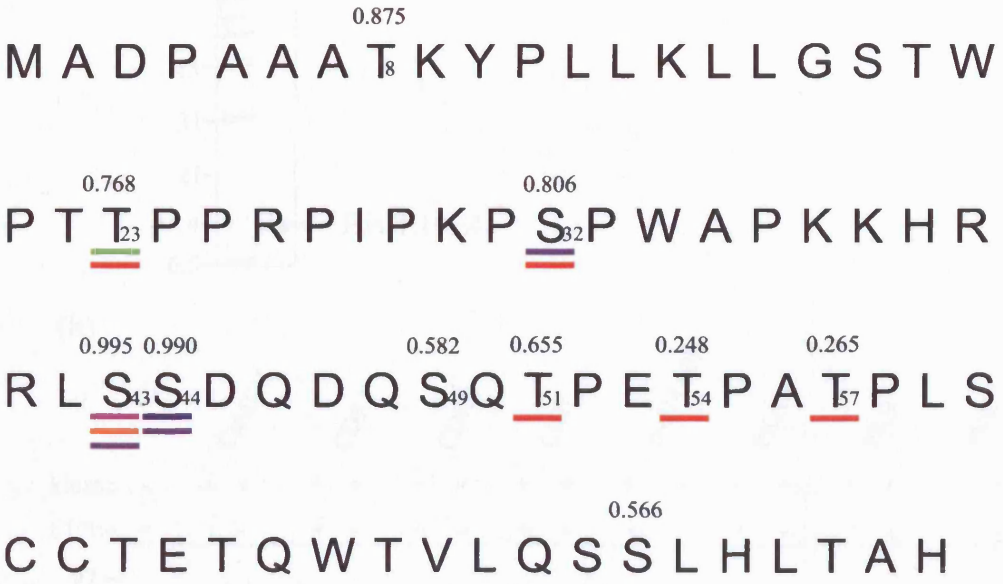
48 92

QSQTPETPATPLSCCTETQWTVLQSSLHLTAHTKDGLTVIVTLHP

3.3 His-E1^E4 is phosphorylated *in vitro* by CDK1, CDK2, p42MAPK, PKA and PKCα

In vitro kinase assays were performed using 1 μg of His-E1^E4 as the substrate (Figure 3.4a) and a range of commercially available recombinant protein kinases; CDK1, CDK2, CKII, CaMKII, p42MAPK, PKA, PKB and PKCα. Radioactive ATP (γ ^{32}P-ATP) was incorporated into the reaction, allowing detection of any 16E1^E4 phosphorylation. SDS-PAGE was performed to separate 16E1^E4 and kinase and the dye front was run off to ensure that free ^{32}P-ATP was removed from the gel. The gel was dried and a PhosphorImager used to detect, phosphorylated protein. Phosphorylated 16E1^E4 was seen with CDK1, CDK2, p42MAPK, PKA and PKCα kinase assays (Figure 3.4b). Interestingly, p42MAPK phosphorylation caused a gel-shift from 14.3 kDa to approximately 16 kDa that was clearly visibly in a 15 % acrylamide gel (Figure 3.4b and c). Note that in this study, all gels showing 16E1^E4 are 15 % gels since the gel-shift is difficult to visualise in a lower percentage gel. Non-16E1^E4 bands were seen with CKII and PKCα kinase assays with apparent molecular weights of 40 kDa and 80 kDa respectively (Figure 3.4b). These represented autophosphorylated CKII (Litchfield et al., 1991; Palen and Traugh, 1991) and PKCα (Liu and Heckman, 1998; Newton, 1995) respectively.

To ensure that CaMKII, CKII and PKB can phosphorylate substrates under these kinase assay conditions, they were tested for their ability to phosphorylate 1 μg of their known substrates *in vitro*. Bovine myelin basic protein (apparent molecular mass ~20 kDa) was phosphorylated by CaMKII, bovine α casein (apparent molecular mass ~30 kDa) was phosphorylated by CKII and a glycogen synthase kinase-3 (GSK-3) fusion protein (apparent molecular mass ~30 kDa) was phosphorylated by PKB (Figure 3.4d). This confirmed that these kinases are functional in these conditions, therefore 16E1^E4 must be a poor substrate for them.



Kinase	Consensus
CDK	S/T-P-X-R/K
CKII	S/T-X-X-D/E
CaMKII	R-X-X-S/T
PKA	R-R/K-X-S/T, R-X-S/T
PKC	K/R-X-X-S/T, K/R-X-S/T
MAPK	P-X-S/T-P, X-X-S/T-P

Figure 3.3 Protein kinase consensus sites in 16E1^E4
The 16E1^E4 amino acid sequence is shown and serines/threonines that fall in a protein kinase consensus site are underlined by a coloured line. Each colour corresponds to a different kinase; the key is shown in the table above. The table also shows the full consensus site for each kinase. The amino acid number (in the context of the 16E1^E4 sequence) for each underlined amino acid is indicated as a subscript. All the amino acids with a NetPhos score above 0.5 are indicated with their NetPhos scores aligned above them.

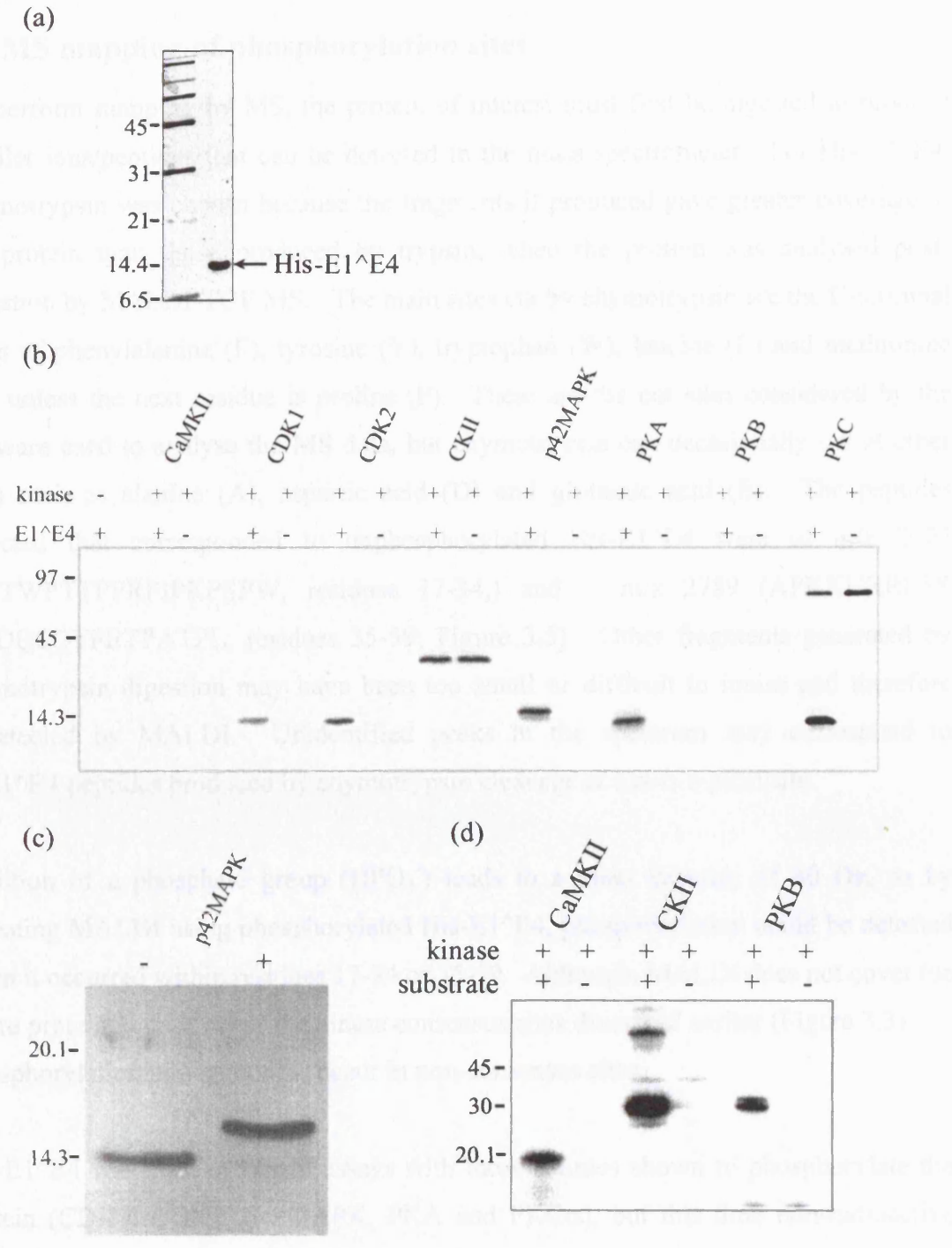


Figure 3.4 *In vitro* phosphorylation of His-E1^E4

(a) Silver stain following SDS-PAGE of purified unphosphorylated His-E1^E4. (b) Autoradiograph of samples separated by SDS-PAGE following *in vitro* kinase assays with γ ^{32}P-ATP. His-E1^E4 (1 µg) was used as the substrate in CDK1, CDK2, p42MAPK, PKA and PKCα kinase assays. (c) Silver stain following SDS-PAGE of His-E1^E4 that had been subjected to kinase assay conditions in the presence (+) and absence (-) of p42MAPK. (d) Autoradiograph following SDS-PAGE of kinase assay samples; reactions were performed to confirm that CaMKII, CKII and PKB can phosphorylate their known substrates *in vitro*. The substrates used for CaMKII, CKII and PKB were 1 µg bovine myelin basic protein, bovine α casein and a GSK-3 fusion protein respectively.

3.4 MS mapping of phosphorylation sites

To perform mapping by MS, the protein of interest must first be digested to produce smaller ions/peptides that can be detected in the mass spectrometer. For His-E1^E4, chymotrypsin was chosen because the fragments it produced gave greater coverage of the protein than those produced by trypsin, when the protein was analysed post-digestion by MALDI-TOF MS. The main sites cut by chymotrypsin are the C-terminal sides of phenylalanine (F), tyrosine (Y), tryptophan (W), leucine (L) and methionine (M) unless the next residue is proline (P). These are the cut sites considered by the software used to analyse the MS data, but chymotrypsin can occasionally cut at other sites such as alanine (A), aspartic acid (D) and glutamic acid (E). The peptides detected that corresponded to unphosphorylated His-E1^E4 were of m/z 2002 (GSTWPTTPPRPIPKPSPW, residues 17-34,) and m/z 2789 (APKKHRRLSS DQDQSQTPETPATPL, residues 35-59; Figure 3.5). Other fragments generated by chymotrypsin digestion may have been too small or difficult to ionise and therefore undetected by MALDI. Unidentified peaks in the spectrum may correspond to 16E1^E4 peptides produced by chymotrypsin cleavage at a non-typical site.

Addition of a phosphate group (HPO_3^-) leads to a mass increase of 80 Da, so by repeating MALDI using phosphorylated His-E1^E4, phosphorylation could be detected when it occurred within residues 17-34 or 35-59. Although, MALDI does not cover the entire protein, it does cover the kinase consensus sites described earlier (Figure 3.3). Phosphorylation may of course occur in non-consensus sites.

His-E1^E4 was used in kinase assays with those kinases shown to phosphorylate the protein (CDK1, CDK2, p42MAPK, PKA and PKCα), but this time non-radioactive ATP was used. The resultant phosphorylated protein was isolated following electrophoresis by colloidal Coomassie staining and excision of the band. In-gel chymotrypsin digestion was performed to fragment the phosphorylated 16E1^E4 into peptides which were then analysed by MS. Phosphorylation by CDK1 or CDK2 gave an ion of m/z ~2082, corresponding to addition of one phosphate to the peptide GSTWPTTPPRPIPKPSPW (Figure 3.6a). PKA, PKCα or p42MAPK phosphorylation resulted in the ion of m/z ~2868, corresponding to addition of one phosphate to the peptide APKKHRRLSSDQDQSQTPETPATPL (Figure 3.6b). The presence of more

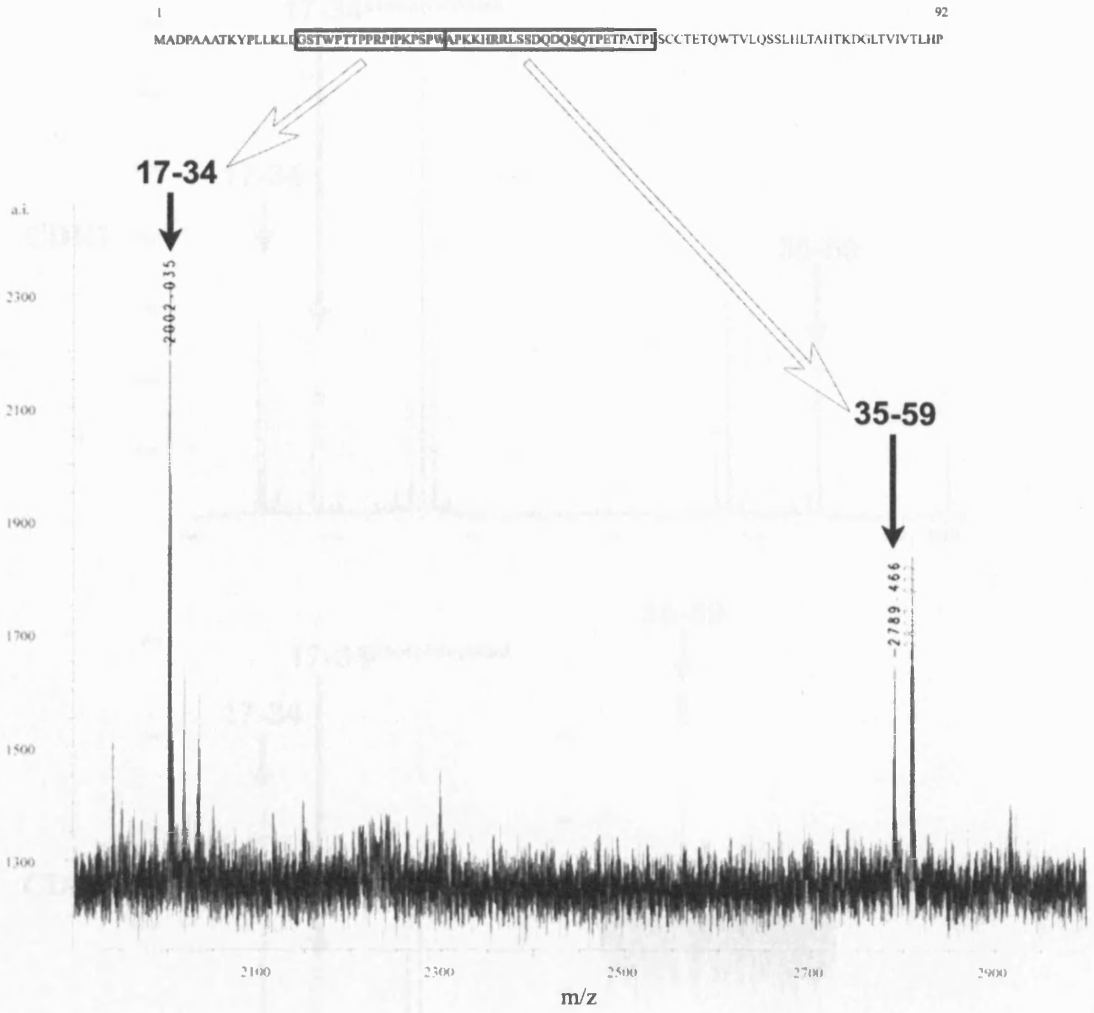


Figure 3.5 MALDI of His-E1^E4

Purified bacterially-expressed His-E1^E4 was cleaved with chymotrypsin by in-gel digestion. The sample was analysed by MALDI-TOF MS and the resulting spectrum is shown. Ions of m/z 2002 and 2789, corresponding to E1^E4 peptides, are indicated (the residue numbers are also shown).

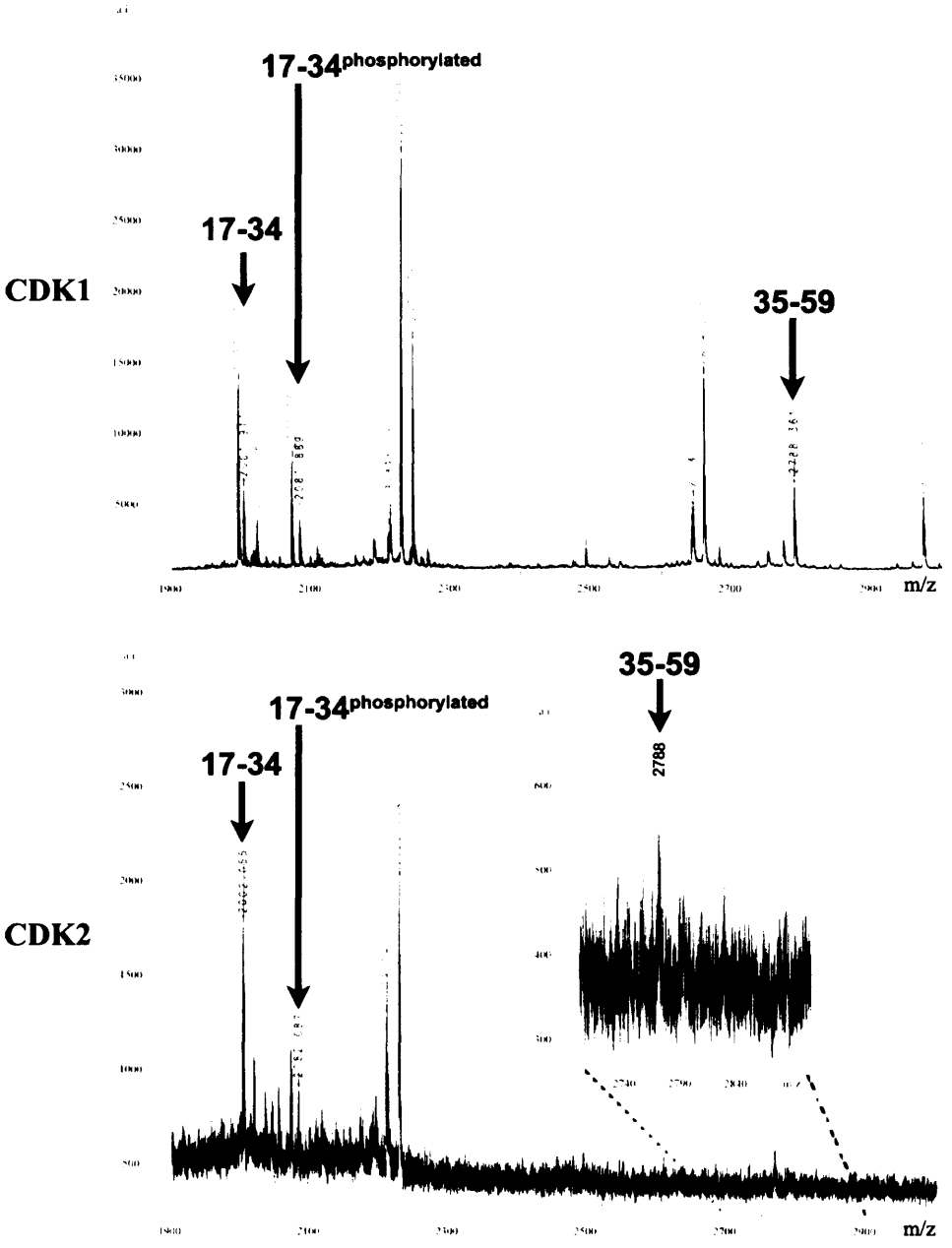
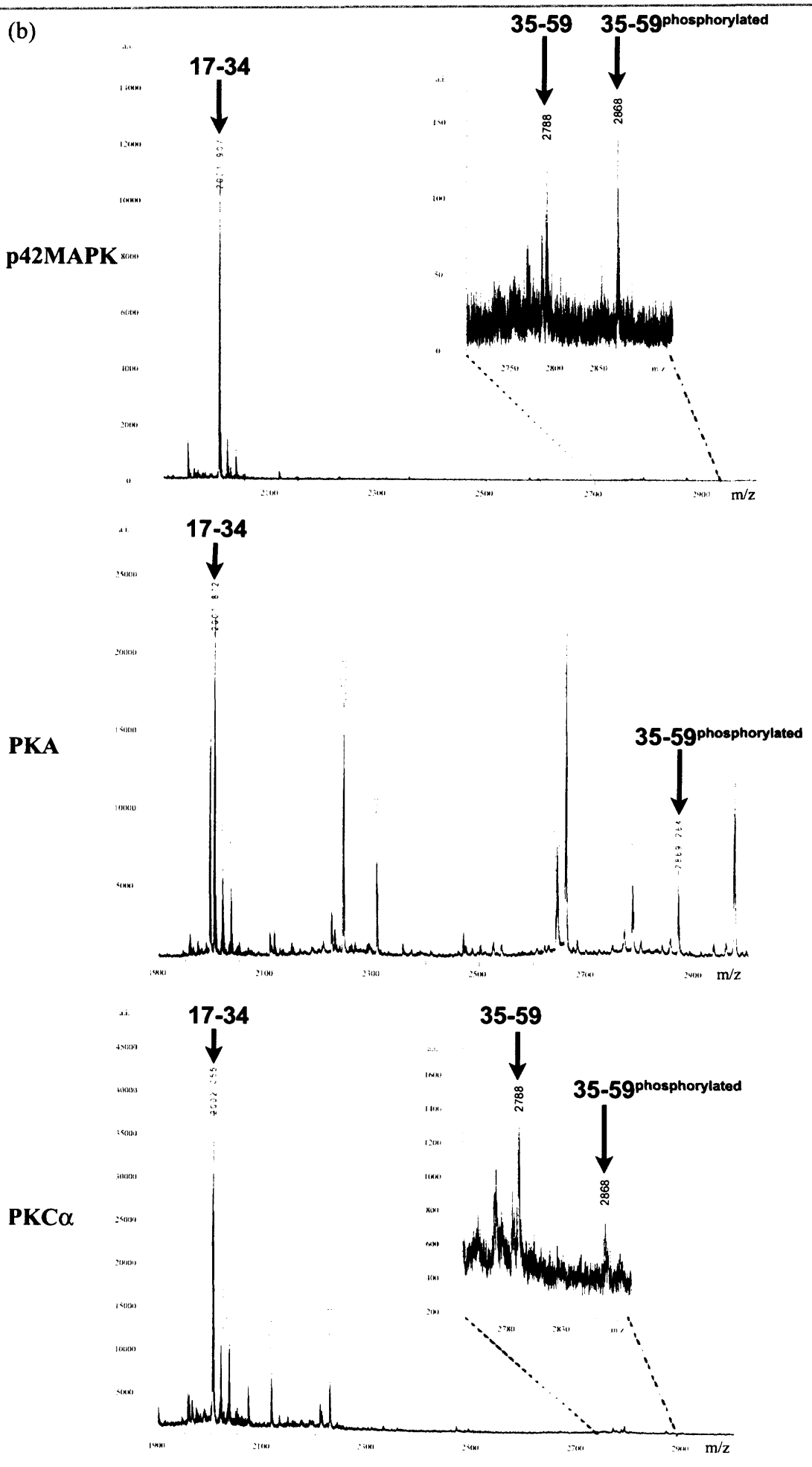


Figure 3.6 MALDI of phosphorylated His-E1^E4
Purified His-E1^E4 was phosphorylated *in vitro* by (a) CDK1 and CDK2, and (b) p42MAPK, PKA and PKCα. The phosphorylated His-E1^E4 was digested by chymotrypsin and analysed by MALDI. E1^E4 ions are indicated in each MALDI spectrum.

(b)


p42MAPK
17-34
35-59
35-59phosphorylated
2788
2868
m/z
PKA
17-34
35-59phosphorylated
m/z
PKCα
17-34
35-59
35-59phosphorylated
2788
2868
m/z

than one phosphorylation site within these ions with any single kinase assay was never detected by MALDI.

Nanospray coupled with collision-induced fragmentation was used to map the exact residues phosphorylated by the kinases. This approach was a success with His-E1^E4 phosphorylated by CDK1 and by PKA. The phosphopeptides obtained by phosphorylation by CDK2, p42MAPK and PKCα were too weak to be detected by nanospray.

His-E1^E4 was phosphorylated *in vitro* and samples prepared for MS and digested with chymotrypsin. The full-scan nanospray spectrum of CDK1-phosphorylated His-E1^E4 indicated an ion of m/z 1041.6. This corresponded to the mono-phosphorylated peptide, GSTWPTTPPRPIPKPSPW (residues 17-34), with a mass of 2082 Da but with a charge of +2, hence a m/z of 1041.6 (Figure 3.7). This ion was selected for the ion trap where it was further fragmented (a process called MS^2). Fragmentation occurs along the peptide backbone, each amino acid successively fragmenting off, both in the N- to C-terminus direction to leave 'y ions' and in the C- to N-terminus direction to leave 'b ions'. After ion 1041.6 was subjected to MS^2 several b and y ions were detected (Figure 3.8).

The ions of significance included the b_{14} ion (the subscript is the number of amino acids in the ion) of m/z 1516.9 corresponding to the unphosphorylated fragment, GSTWPTTPPRPIPK, and the y_4 ion of m/z 566.1 corresponding to the phosphorylated peptide, PSPW. This revealed that serine 32 is phosphorylated by CDK1. Another indication that 1041.6 contained a phosphate is the loss of phosphoric acid (H_3PO_4; 98 Da) during fragmentation. The +2 charged ion of m/z 1041.6 lost phosphoric acid to leave the +1 charged ion at m/z 993. To confirm that serine 32 is the phosphorylation site, the y_4 ion (m/z 566.1) was further fragmented (MS^3). This produced a y ion of m/z 469.1 corresponding to the phosphorylated fragment, SPW and an ion at m/z 468.1 corresponding to loss of phosphoric acid from PSPW (Figure 3.9).

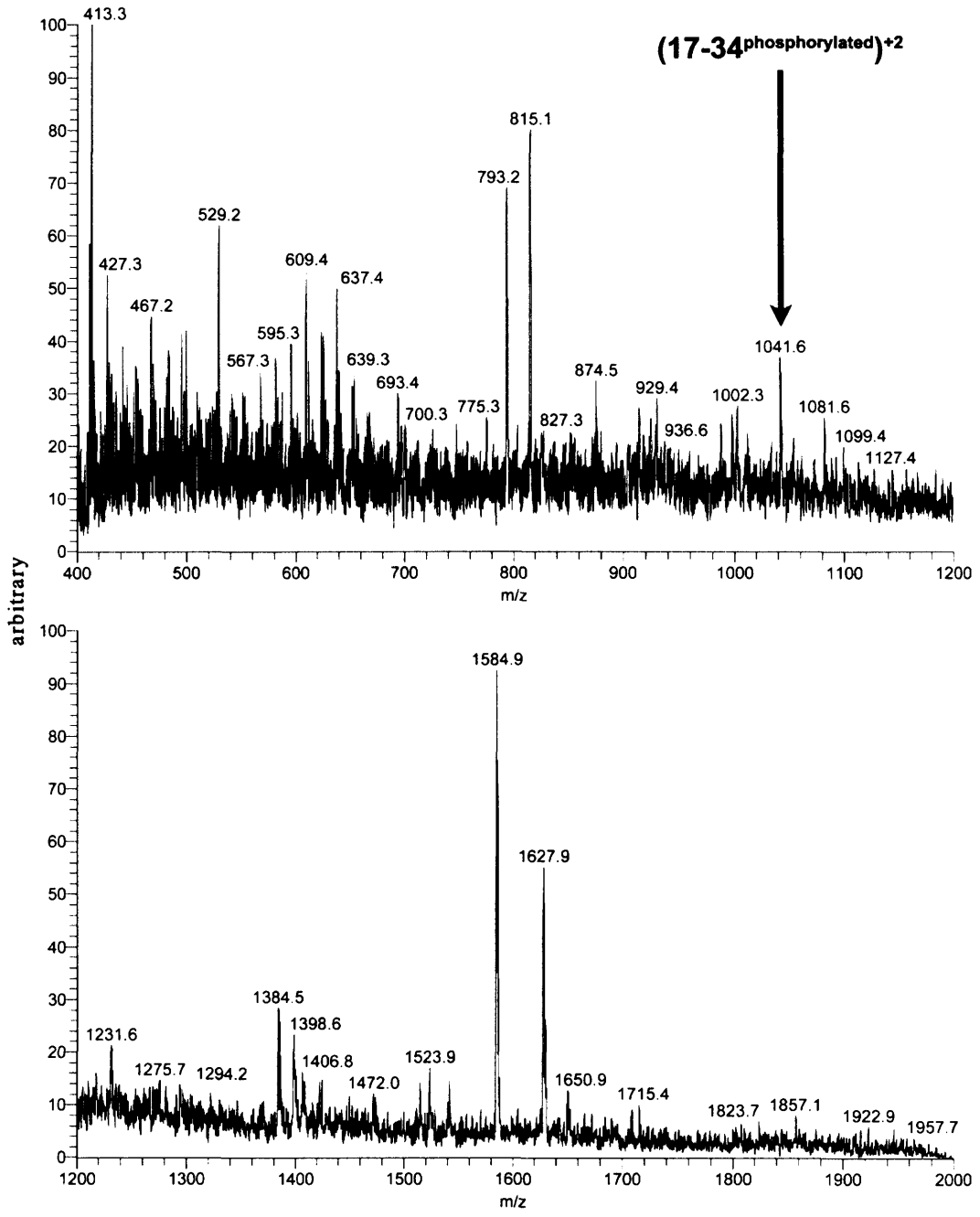


Figure 3.7 Nanospray of CDK1-phosphorylated His-E1^E4
Purified His-E1^E4 was phosphorylated *in vitro* by CDK1, separated by SDS-PAGE and in-gel digested with chymotrypsin. The sample was analysed by nanospray MS. A peak at m/z 1041.6 corresponding to an E1^E4 peptide is indicated. This ion was then selected for collision-induced fragmentation in the ion trap.

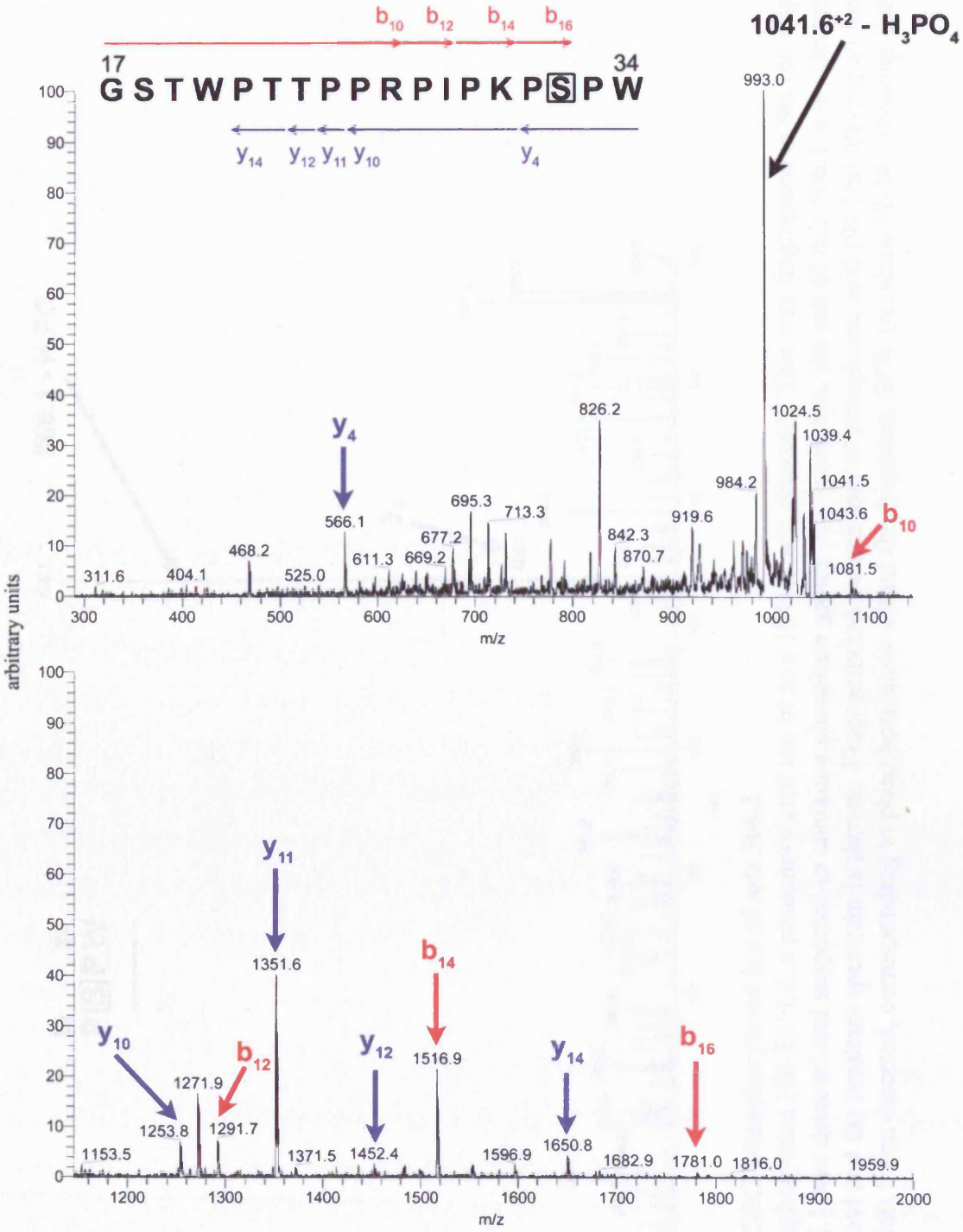


Figure 3.8 Fragmentation of the CDK1-phosphorylated ion of m/z 1041.6
Following nanospray of CDK1-phosphorylated His-E1^E4, a phosphorylated ion of m/z 1041.6 was detected and this was fragmented in the ion trap. The spectrum shows the b and y ions detected after fragmentation. Above the spectrum is a diagram of the peptide sequence fragmented with the corresponding b and y ions aligned. The b and y ions revealed that serine 32 (indicated in a box) was phosphorylated by CDK1. Also indicated is an ion of m/z 993 corresponding to the +2 ion of m/z 1041.6 losing a 98 Da phosphoric acid.

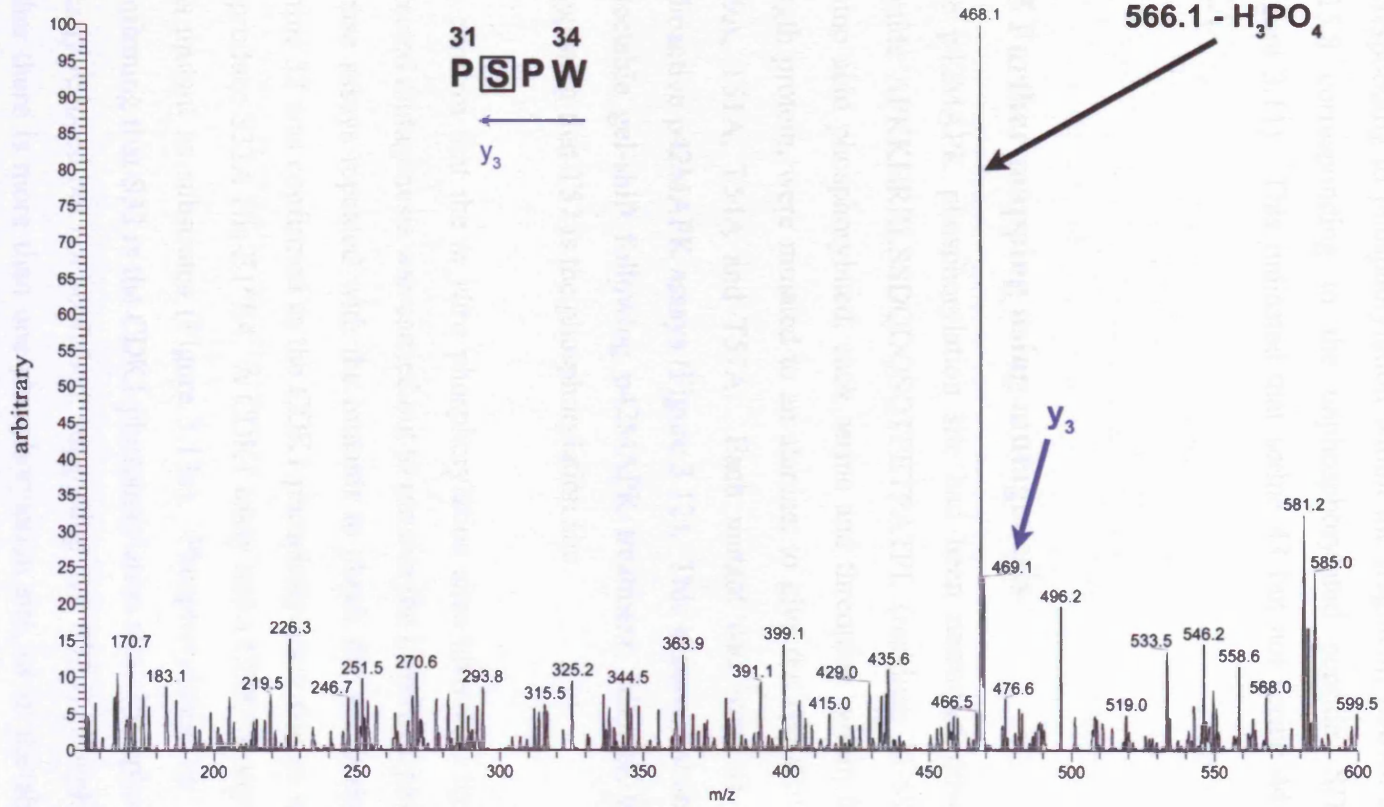


Figure 3.9 Fragmentation of the CDK1-phosphorylated ion of m/z 566.1

Following nanospray of CDK1-phosphorylated His-E1^E4, a phosphorylated ion of m/z 1041.6 was detected. This was fragmented in the ion trap and subsequently, an ion of m/z 566.1 was detected and suspected to contain a phosphate group. To verify this, the ion of m/z 566.1 was selected for the ion trap and further fragmented and the resultant spectrum is shown. Fragmentation caused loss of phosphoric acid (98 Da) leaving an ion of m/z 468.1. Also a y ion of m/z 469.1 was detected, corresponding to phosphorylation within the fragment, SPW (residues 32-34), showing that serine 32 was

The full-scan nanospray spectrum of PKA-phosphorylated His-E1^E4 showed an ion of m/z 1154.6 which corresponded to the +2 form of the mono-phosphorylated peptide, RRLSSDQDQSQTPETPATPL (Figure 3.10). This was selected for the ion trap for MS^2. The significant ions detected post-fragmentation include the b_4 ion of m/z 593.2 corresponding to phosphorylation within the fragment, RRLS, and the y_{16} ion of m/z 1715.8 corresponding to the unphosphorylated peptide, SDQDQSQTPETPATPL (Figure 3.11). This indicated that serine 43 but not serine 44 is phosphorylated by PKA.

3.5 Further mapping using mutagenesis

The p42MAPK phosphorylation site had been narrowed down by MALDI to the peptide APKKHRRLSSDQDQSQTPETPATPL (residues 35-59). To find the exact amino acid phosphorylated, each serine and threonine within this region of the full-length protein, were mutated to an alanine, to give the His-E1^E4 mutants, S43/44A, S49A, T51A, T54A and T57A. Each mutant was used as the substrate in non-radioactive p42MAPK assays (Figure 3.12). This experiment showed that T57 had no detectable gel-shift following p42MAPK treatment, whereas the other mutants did, suggesting that T57 is the phosphorylation site.

To confirm that the *in vitro* phosphorylation sites identified by MS are correct, site-directed mutagenesis was carried out to remove the identified phosphorylation sites and kinase assays repeated with the mutants to check for abrogation of phosphorylation. Serine 32 was confirmed as the CDK1 phosphorylation site so was changed to alanine to produce S32A His-E1^E4. A CDK1 assay and a CDK2 assay were performed using this mutant as substrates (Figure 3.13a). Phosphorylation by CDK1 was abolished, confirming that S32 is the CDK1 phosphorylation site. Phosphorylation by CDK2 was clearly reduced but not abolished, suggesting that S32 is phosphorylated by CDK2 but either there is more than one phosphorylation site, or in the absence of S32, another amino acid is targeted. By MS, serine 43 was found to be the PKA phosphorylation site, so this residue was mutated to alanine to produce S43A His-E1^E4. This mutant was used in a PKA assay which showed that phosphorylation was reduced but not

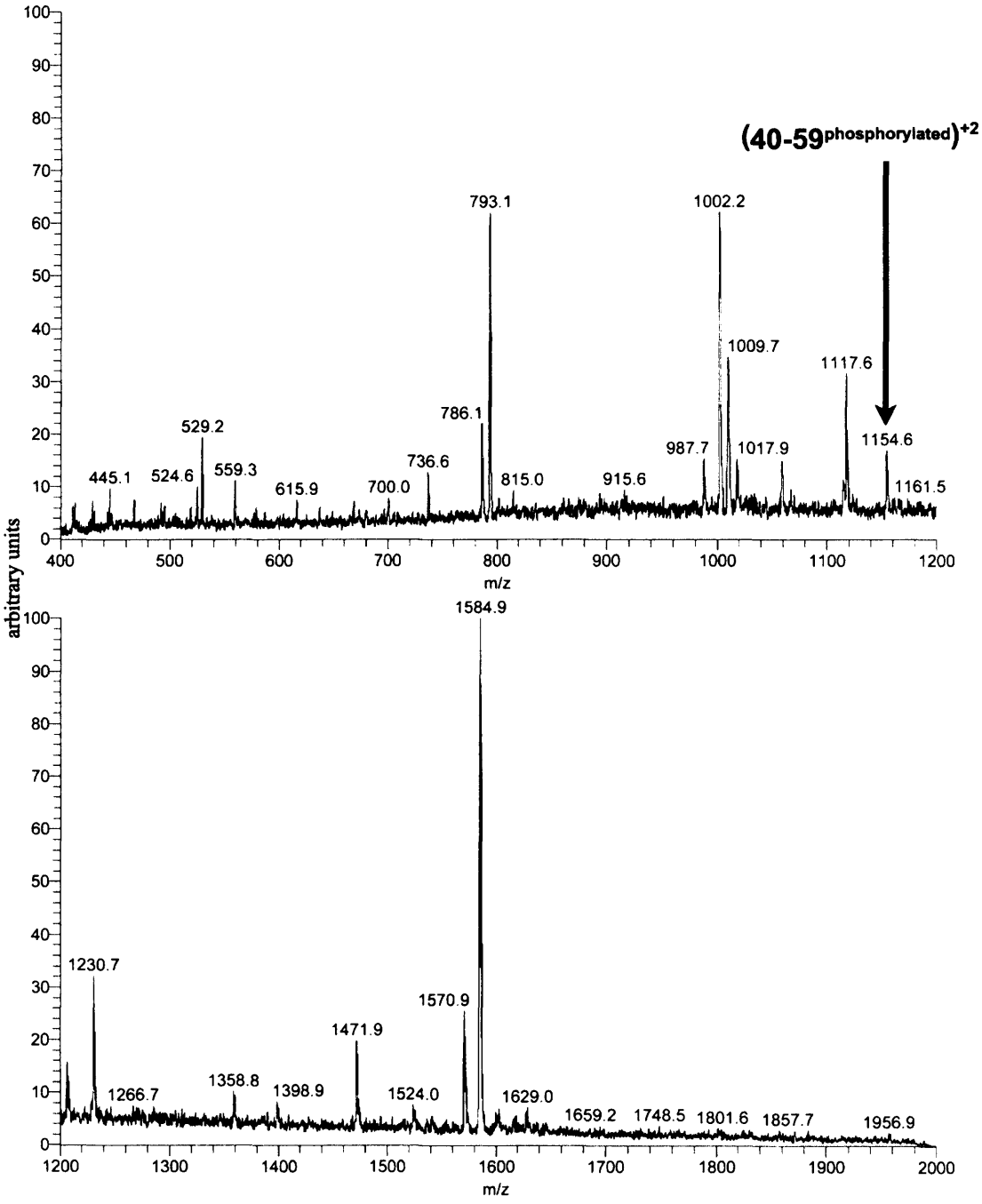


Figure 3.10 Nanospray of PKA-phosphorylated His-E1^E4
Purified His-E1^E4 was phosphorylated *in vitro* by PKA, separated by SDS-PAGE and in-gel digested with chymotrypsin. The sample was analysed by nanospray MS. An ion corresponding to the +2 form of mono-phosphorylated RRLSSDQDQSQ TPETPATPL was detected at m/z 1154.6. This was selected for fragmentation in the ion trap.

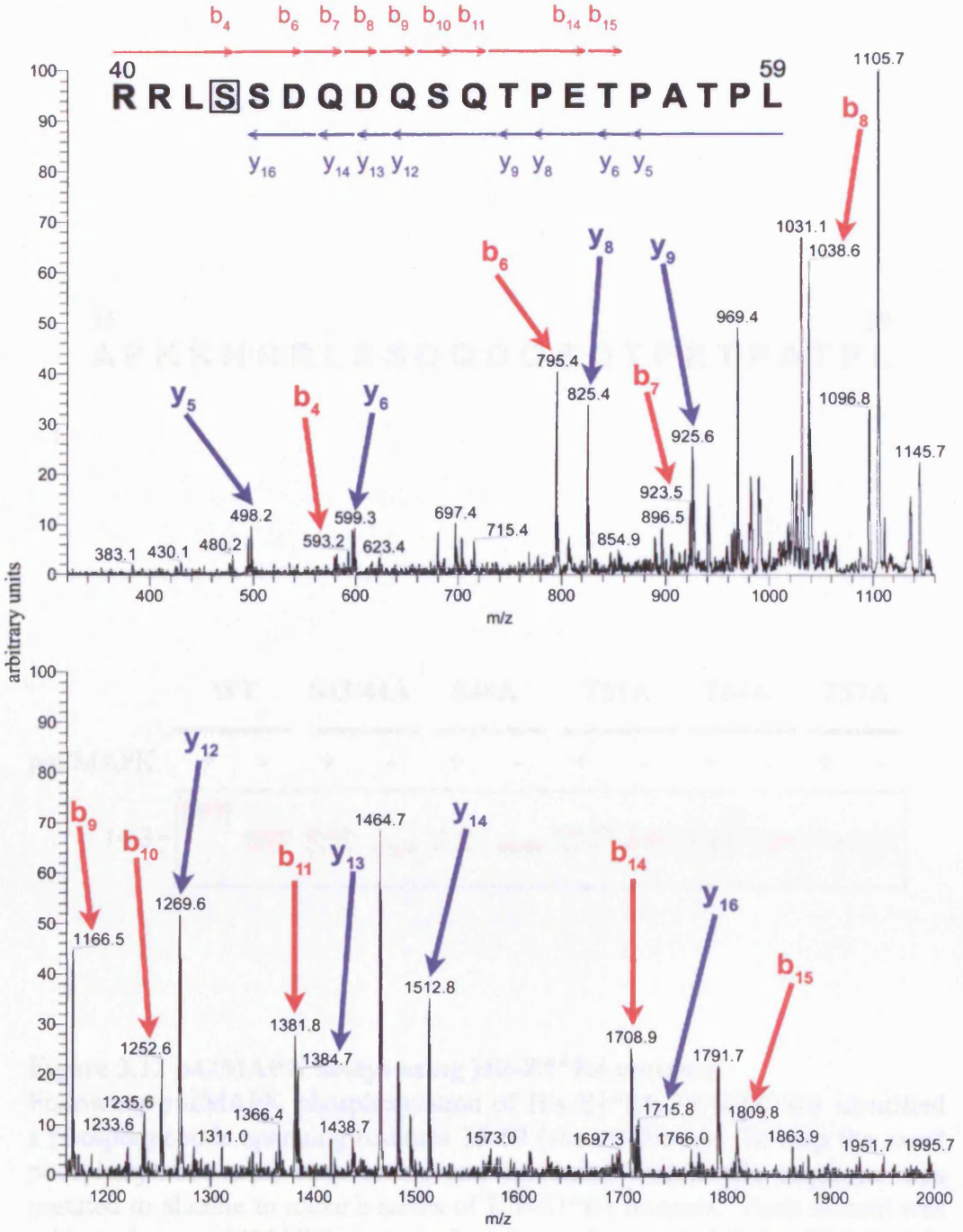


Figure 3.11 Fragmentation of the PKA-phosphorylated ion of m/z 1154.6
Nanospray of PKA-phosphorylated His-E1^E4 allowed detection of a phosphorylated +2 ion of m/z 1154.6 which was then fragmented in the ion trap. The spectrum shows the b and y ions detected after fragmentation. Above the spectrum is a diagram of the peptide sequence that was fragmented with the corresponding b and y ions aligned. The b and y ions indicated that serine 43 (shown in a box) was phosphorylated by PKA and no other phosphorylated residues were detected.

35 59

APKKHRRLSSDQDQSQTPETPATPL

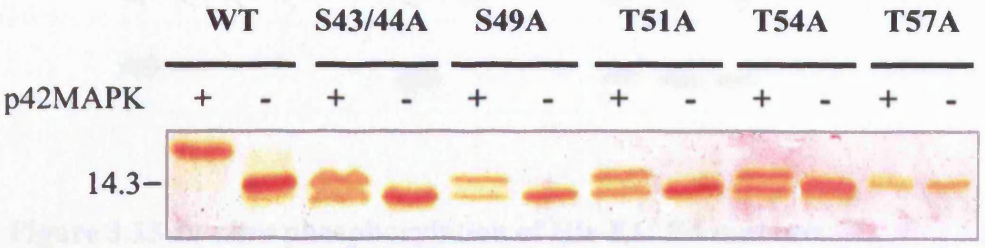


Figure 3.12 p42MAPK assays using His-E1^E4 mutants
Following p42MAPK phosphorylation of His-E1^E4, MALDI MS identified a phosphopeptide spanning residues 35-59 (shown above). To map the exact phosphorylation site, each serine and threonine within this sequence was mutated to alanine to make a series of His-E1^E4 mutants. Each mutant was subjected to a p42MAPK assay and analysed for a gel-shift by SDS-PAGE, followed by silver staining.

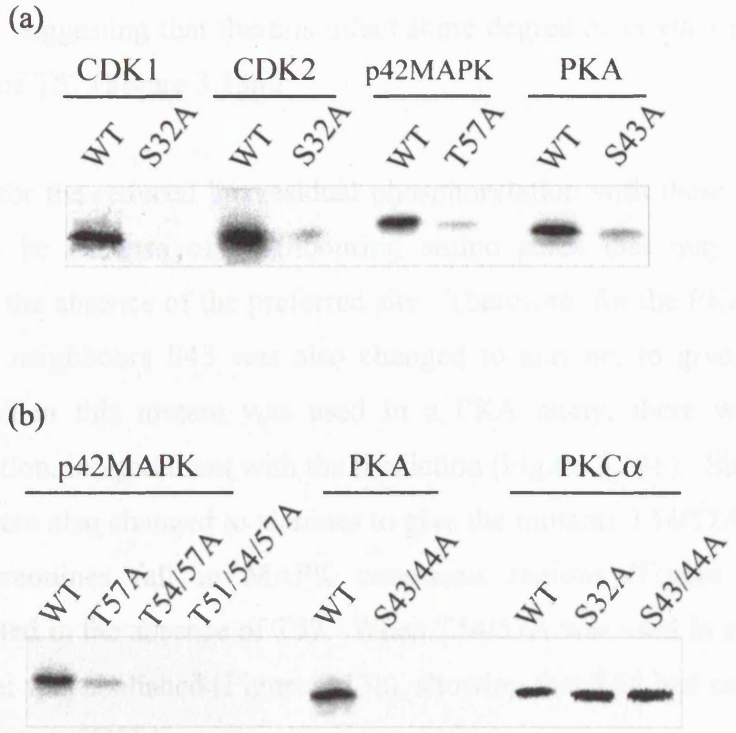


Figure 3.13 *In vitro* phosphorylation of His-E1^E4 mutants
(a) Autoradiograph of CDK1, CDK2, p42MAPK and PKA *in vitro* kinase assays using WT and mutant His-E1^E4 proteins. The mutant proteins had the phosphorylation sites (as detected by MALDI / mutagenesis) mutated by replacing the serine/threonine with an alanine. (b) Further sites were mutated in an attempt to abolish phosphorylation by p42MAPK and PKA; autoradiograph following kinase assays is shown. PKC consensus site mutants (S32A and S43/44A) were also tested in PKCα kinase assays.

abolished (Figure 3.13a). Similarly, the T57A mutant that previously showed no p42MAPK-induced gel-shift (Figure 3.12), did incorporate some ATP in a radioactive kinase assay suggesting that there is infact some degree of i*n vitro* phosphorylation in the absence of T57 (Figure 3.13a).

The reason for the reduced but residual phosphorylation with these kinase assays was predicted to be because of neighbouring amino acids that may act as phosphate acceptors in the absence of the preferred site. Therefore, for the PKA assay, the serine residue that neighbours S43 was also changed to alanine, to give a double mutant, S43/44A. When this mutant was used in a PKA assay, there was no longer any phosphorylation, in agreement with the prediction (Figure 3.13b). Similarly, threonines 51 and 54 were also changed to alanines to give the mutants T54/57A and T51/54/57A, as these threonines fall in MAPK consensus regions (Figure 3.3) so may be phosphorylated in the absence of T57. When T54/57A was used in a p42MAPK assay, the ^{32}P signal was abolished (Figure 3.13b), showing that T54 had caused the low level phosphorylation of T57A.

A PKCα phosphorylation site had been mapped to the peptide APKKHRRLSSDQD QSQTPETPATPL (residues 35-59) by MALDI. 16E1^E4 has three consensus sites for PKC, at serine 32, serine 43 and serine 43, so the latter two fall within this peptide. The S32A mutant and S43/44A mutant were tested in PKCα assays, however both retained wild-type levels of phosphorylation (Figure 3.13b). PKCα may therefore target all consensus sites, or another site within this peptide.

3.6 Discussion

This study has demonstrated that 16E1^E4 has several candidate phosphorylation sites and CDK1, CDK2, PKA, PKCα and p42MAPK can phosphorylate bacterially-expressed 16E1^E4 *in vitro*. Interestingly, phosphorylation by p42MAPK causes a gel-shift of His-E1^E4, suggesting a structural change. The *in vitro* phosphorylation sites have been mapped for some of these kinases. CDK1 and CDK2 phosphorylate at serine 32, PKA phosphorylates at serine 43 and p42MAPK phosphorylates at threonine 57. It has been shown, however, that if these sites are mutated the corresponding kinase can phosphorylate other sites *in vitro*. This must be considered when producing phosphorylation mutants for functional analysis, for example, to ensure total abrogation of PKA phosphorylation, both serines 43 and 44 should be mutated, since serine 44 appears to be phosphorylated in the absence of serine 43.

The phosphorylation sites of PKA and p42MAPK fit the classical consensus sites for these kinases, but the CDK1 and CDK2 phosphorylation site at serine 32 does not fit the S/T-P-X-R/K consensus as it lacks the R/K residue. Surprisingly, there was no detection of phosphorylation at threonine 23 by CDK1 even though this falls in the only CDK consensus site present. This is supported by the absence of phosphorylation in the peptide of residues 17-30 (m/z 1516.9) after collision-induced fragmentation of the phosphorylated peptide (residues 17-34, m/z 1041.6). Threonine 23 was shown to be required for E1^E4-induced G2 arrest in *S.pombe* since a mutation to alanine abolished arrest, however, mutating to the phosphate mimics, glutamic or aspartic acid, did not rescue arrest (Davy et al., 2002). This also suggests that threonine 23 phosphorylation may not be important.

Phosphorylation by CDK at a non-consensus site is not uncommon and has been reported for several proteins, for example poly(A) polymerase (Colgan et al., 1998), ribosomal S6 kinase 1 (Shah et al., 2003) and cytoplasmic polyadenylation-element-binding protein (CPEB; Thom et al., 2003). CDK substrate interaction is believed to depend on bipartite motif recognition of the substrate; a cyclin-binding motif and the phosphorylation site (Takeda et al., 2001). The existence of two types of substrate have been proposed; the first is a high turnover substrate that has a consensus phosphorylation site but binds weakly to cyclin/CDK, the second is a low turnover

substrate which binds tightly to cyclin/CDK but has a non-consensus phosphorylation site (Murray, 2004). 16E1^E4 may prove to be in the latter group.

The MS method for phosphorylation mapping is frequently used, however, it is difficult to achieve total coverage of the protein. In this case, only residues 17-59 were covered out of the 92 amino acids of 16E1^E4. The low level phosphorylation of the S32A mutant by CDK2 and the phosphorylation of the S32A and S43/44A mutants by PKC could therefore also be explained by an additional phosphorylation site in a region not covered by MALDI. The existence of phosphorylation sites outside these regions was disproved for CDK1, PKA and p42MAPK because mutagenesis within these regions abolished phosphorylation by these kinases. The other problem with MS is that phosphorylated ions rarely give intense peaks, as phosphorylation makes peptides negatively charged causing weak ionisation in the positive mode in the mass spectrometer. The absence of strong peaks for some of the phosphorylation events prevented successful phosphorylation mapping by nanospray coupled with collision-induced fragmentation.

The phosphorylation events described in this chapter are of great interest and value, however, these events occured *in vitro* where the conditions are perhaps more favourable for phosphorylation than they are *in vivo*. For example, the kinase concentrations were high and phosphatases or any other inhibitory factors were absent. It is therefore important to analyse 16E1^E4 phosphorylation in mammalian cell culture or *in vivo*. The next chapter describes phosphorylation events of 16E1^E4 when it is expressed in SiHa cells.

Chapter 4: Analysis of 16E1^E4 Phosphorylation Sites in Cell Culture

4.1 Introduction

Following the mapping of *in vitro* phosphorylation sites of 16E1^E4 in Chapter 3, it was important to determine the *in vivo* phosphorylation sites. The extraction and analysis of 16E1^E4 from infected lesions is difficult especially if the tissue has been formalin- fixed. Instead, the protein was expressed and analysed in cell culture. The main aim was to determine which (if any) of the *in vitro* phosphorylation sites are phosphorylated when the protein is expressed in cultured cells. The 16E1^E4 protein was expressed in SiHa cells using recombinant adenovirus (rAd) infection or plasmid transfection. SiHa cells are a cervical cancer cell line, containing integrated HPV16 DNA and expressing E6 and E7 but not E1^E4. Being an HPV-transformed cell line, SiHa cells are a good choice of cell type to analyse E1^E4 post-translational modifications and activities.

The approaches used to investigate 16E1^E4 phosphorylation in cells included the use of lambda (λ) phosphatase, the phosphatase inhibitor, okadaic acid (OA) and protein kinase inhibitors. Detection of phosphorylated 16E1^E4 was attempted using phosphotyrosine-, phosphoserine- and phosphothreonine-specific antibodies. The use of such antibodies is potentially a much simpler method for phosphoamino acid analysis compared to traditional techniques involving amino acid hydrolysis (see 3.1.1.1). Isoelectric focusing (IEF; see 4.1.1) was a technique used to separate differentially phosphorylated forms of 16E1^E4 and was found to be useful for purification of the protein for phosphorylation mapping by MALDI. In addition, site-directed mutagenesis was used to remove phosphorylation sites proposed in Chapter 3 from the *in vitro* kinase assays. The mutant 16E1^E4 proteins were then analysed for their ability to be phosphorylated in cells.

4.1.1 IEF (isoelectric focusing) and isoelectric point (pI)

IEF is a useful tool for analysing the phospho-status of proteins since it allows the isoelectric point (pI) of a protein to be determined. The pI of a protein is the pH at which the protein has no overall charge, and addition of a phosphate group will lower

the pI as phosphates are negatively charged (Hardie, 1999). The theoretical pI can be calculated from the protein sequence. Ionisable groups include the N-terminal residue, lysine, arginine and histidine, which have the potential to be positively charged, and the C-terminal residue, aspartic acid and glutamic acid, which can become negatively charged. The number of each of these ionisable groups must be known to calculate the pI (Bjellqvist et al., 1993). The effect of phosphorylation on the pI can also be estimated. The calculated pI can be inaccurate however, because it considers each ionisable residue in isolation and does not take into account the influence of neighbouring residues on the charge. Table 4.1 shows the theoretical pIs of unphosphorylated and phosphorylated forms of 16E1^E4 as calculated by Scansite (http://scansite.mit.edu/calc_mw_pi.html; Obenauer et al., 2003).

Table 4.1 Scansite pI prediction

Theoretical pIs of unphosphorylated and phosphorylated forms of 16E1^E4 as calculated by Scansite (http://scansite.mit.edu/calc_mw_pi.html).

Number of phosphorylation events	0	1	2	3	4
pI	9.14	8.02	7.03	6.57	6.22

IEF was first introduced in 1975 (Klose, 1975; O'Farrell, 1975). It involves the production of a pH gradient in which proteins can be separated electrophoretically according to pI. The original method for creating a gel with a pH gradient used carrier ampholytes (small organic molecules with various isoelectric points and high buffering capacities). When a voltage is applied across an ampholyte mixture, they migrate in the gel until they reach their isoelectric point, thus a pH gradient is produced. Due to some limitations of carrier ampholytes (e.g. the poor reproducibility due to drift of ampholytes towards the cathode), the immobilised pH gradient (IPG) was later introduced (Bjellqvist et al., 1982). An IPG gradient is formed by covalently incorporating acidic and basic buffering groups into the acrylamide matrix at the time the gel is cast. Gels with immobilised pH gradients are commercially available. The IPG system was used for all IEF experiments described in this study. Following IEF, proteins are generally separated according to molecular mass by SDS-PAGE. The IEF

step is termed the 'first dimension' and the SDS-PAGE step is termed the 'second dimension,' and the overall technique referred to as two-dimensional SDS-PAGE (2D SDS-PAGE; Figure 4.1).

4.2 16E1^E4 expressed in cell culture separates into different migratory forms by SDS-PAGE

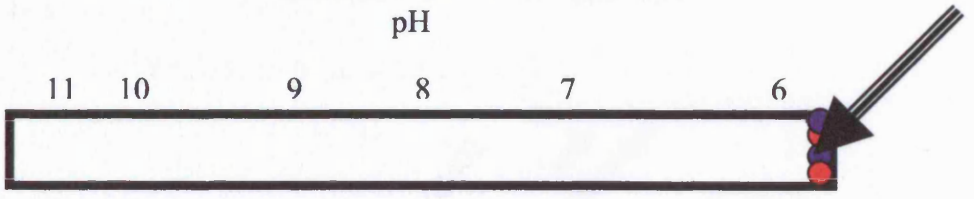
16E1^E4 was expressed in SiHa, NIKS and C33A cells from the CMV promoter of a recombinant adenovirus vector (rAdE1^E4). More than one migratory form was seen after SDS-PAGE with a 15 % acrylamide gel and Western blotting with the anti-16E1^E4 mouse monoclonal, TVG402 (Figure 4.2a). The protein migrated as a 13 kDa band and a 14 kDa band in all three cell lines. However, in C33A cells, the 14 kDa appeared weaker and there was an additional band at ~10 kDa. Probing with an anti-N-terminus antibody failed to detect the 10 kDa form although this antibody detected the 13 kDa band (Figure 4.2b). This suggests that N-terminal cleavage can occur in cell culture, particularly in C33A cells. When monitoring 16E1^E4 expressed in SiHa cells over a 24 h period, Western blotting with TVG402 showed that the 13 kDa band was detected first, followed by the gradual appearance of the 14 kDa band (Figure 4.2c).

4.3 The upper band (14 kDa) of 16E1^E4 is a phosphorylated form

SiHa cells were infected with rAdE1^E4 for 24 h before harvest. The cells were lysed with Empigen and the cell extract treated with/without λ phosphatase. λ phosphatase is a serine/threonine/tyrosine phosphatase from bacteriophage λ (Cohen and Cohen, 1989). The proteins in the cell extract were then separated by SDS-PAGE and 16E1^E4 detected by Western blotting with TVG402 (Figure 4.3a). The Western blot showed that the upper band (14 kDa band) was removed by λ phosphatase. This result suggests that the upper band corresponds to a gel-shift produced as a result of serine, threonine or tyrosine phosphorylation. Interestingly, this gel-shift is similar to that seen following *in vitro* phosphorylation by p42MAPK (Figure 3.4b and c).

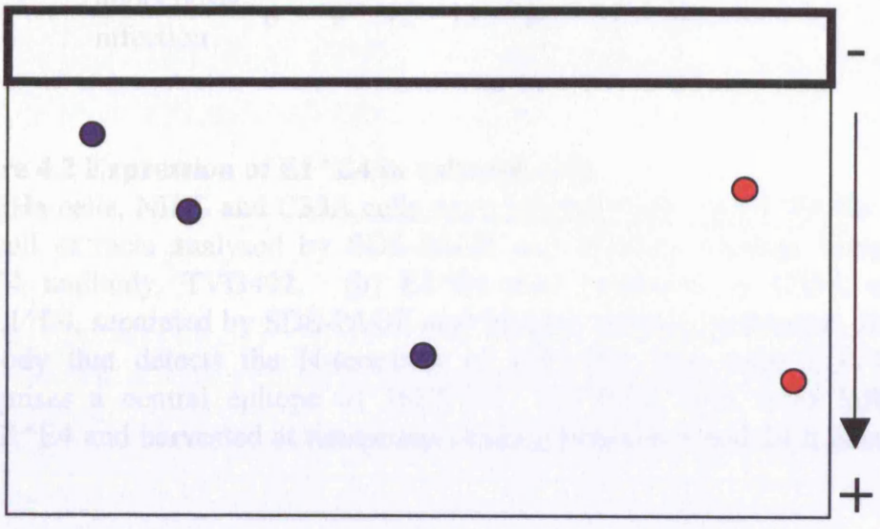
Figure 4.1 Outline of 2D SDS-PAGE
The diagrams below represent the main steps of the 2D SDS-PAGE protocol used in this study. (a) The sample is applied at the acidic end (anode) of a gel strip containing an immobilised pH gradient (IPG). (b) A voltage is applied across the gel. All proteins (basic shown as blue and acidic as red) migrate until they reach the pH at which they have no charge (i.e. their isoelectric point), then isoelectric focusing (IEF) or the first dimension is complete. (c) The second dimension uses SDS-PAGE to separate all the proteins according to size.

(a) Sample application



(b) IEF/ First Dimension

(c) Second Dimension



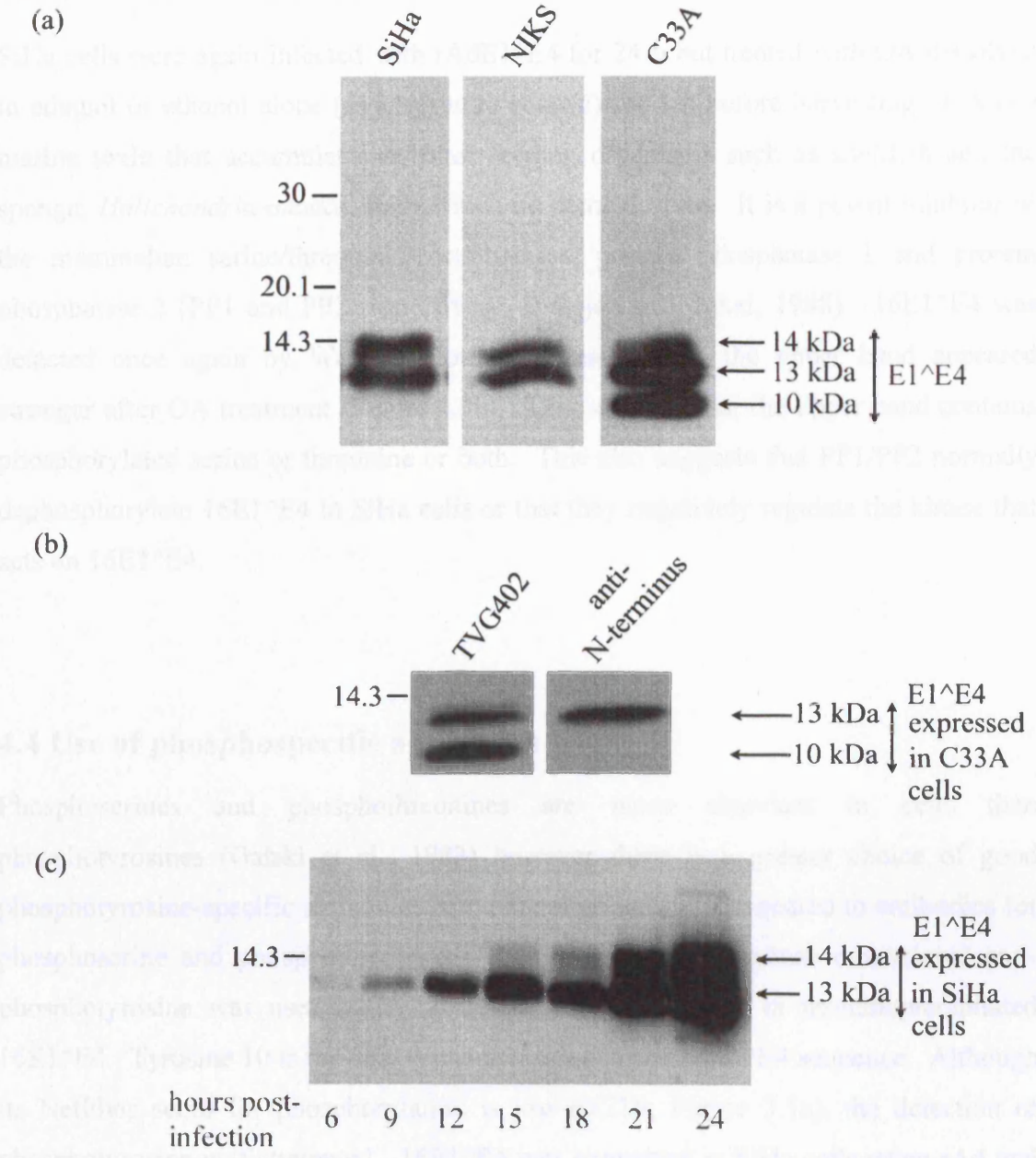


Figure 4.2 Expression of E1^E4 in cultured cells
(a) SiHa cells, NIKS and C33A cells were infected with rAdE1^E4 for 24 h, then the cell extracts analysed by SDS-PAGE and Western blotting using the anti-E1^E4 antibody, TVG402. (b) E1^E4 was expressed in C33A cells using rAdE1^E4, separated by SDS-PAGE and Western blotting performed, first with an antibody that detects the N-terminus of 16E1^E4, then with TGV402 which recognises a central epitope of 16E1^E4. (c) SiHa cells were infected with rAdE1^E4 and harvested at timepoints ranging between 6 and 24 h post-infection.

SiHa cells were again infected with rAdE1^E4 for 24 h but treated with OA dissolved in ethanol or ethanol alone (as a negative control) for 1 h before harvesting. OA is a marine toxin that accumulates in filter feeding organisms such as shellfish and the sponge, *Halichondria okadai*, from which its name derives. It is a potent inhibitor of the mammalian serine/threonine phosphatases, protein phosphatase 1 and protein phosphatase 2 (PP1 and PP2 respectively; Bialojan and Takai, 1988). 16E1^E4 was detected once again by Western blotting, revealing that the upper band appeared stronger after OA treatment (Figure 4.3b). This suggests that the upper band contains phosphorylated serine or threonine or both. This also suggests that PP1/PP2 normally dephosphorylate 16E1^E4 in SiHa cells or that they negatively regulate the kinase that acts on 16E1^E4.

4.4 Use of phosphospecific antibodies

Phosphoserines and phosphothreonines are more abundant in cells than phosphotyrosines (Galski et al., 1983) however there is a greater choice of good phosphotyrosine-specific antibodies commercially available compared to antibodies for phosphoserine and phosphothreonine. The PY20 clone of mouse monoclonal anti-phosphotyrosine was used to try to detect phosphotyrosine in immunoprecipitated 16E1^E4. Tyrosine 10 is the only tyrosine residue in the 16E1^E4 sequence. Although its NetPhos score for phosphorylation is low (0.218; Figure 3.1a), the detection of phosphotyrosine was attempted. 16E1^E4 was expressed in SiHa cells using rAd and was successfully immunoprecipitated as shown by Western blotting with TVG402 (Figure 4.4a). When probed with PY20, however, no band corresponding to E1^E4 was detected (long exposure of the blot led to detection of the light chain of the antibody used for the immunoprecipitation, probably due to non-specific interaction with the anti-mouse secondary antibody) although PY20 did detect active recombinant p42MAPK phosphorylated at tyrosine 185 (Figure 4.4b). Similar experiments were attempted with the mouse monoclonal anti-phosphoserine antibody, clone PSR-45, and the mouse monoclonal anti-phosphothreonine antibody, clone 1E11, however, even after several attempts, the background signal after Western blotting was very high indeed and no conclusions could be made (data not shown).

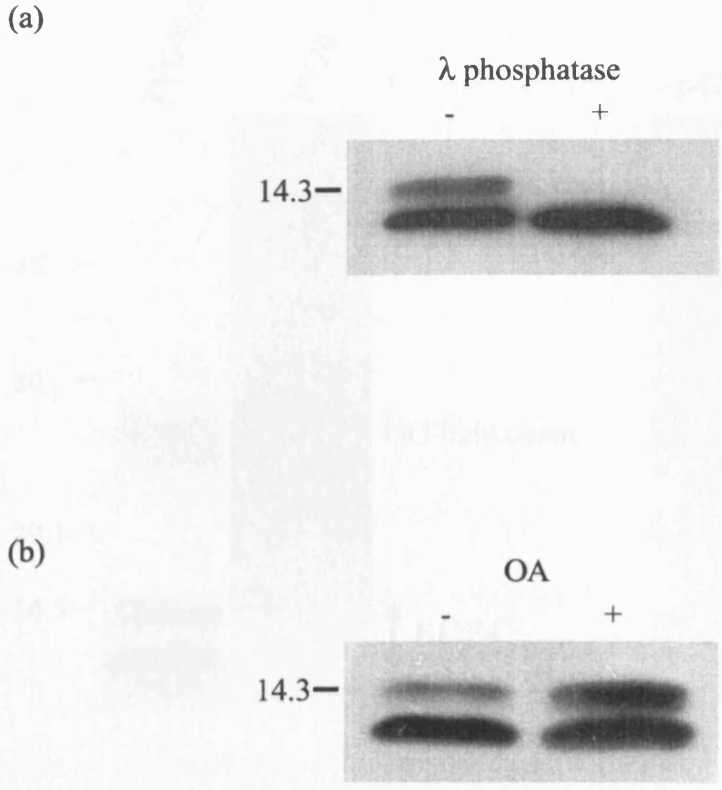


Figure 4.3 Effect of l phosphatase and okadaic acid on E1^E4
(a) Western blot of E1^E4 expressed using rAd in SiHa cells. The cell extracts were treated with (+) or without (-) λ phosphatase prior to SDS-PAGE. (b) Western blot of E1^E4 expressed using rAd in SiHa cells. The cells were treated with (+) or without (-) okadaic acid (OA; a phosphatase inhibitor) for 1 h before harvest.

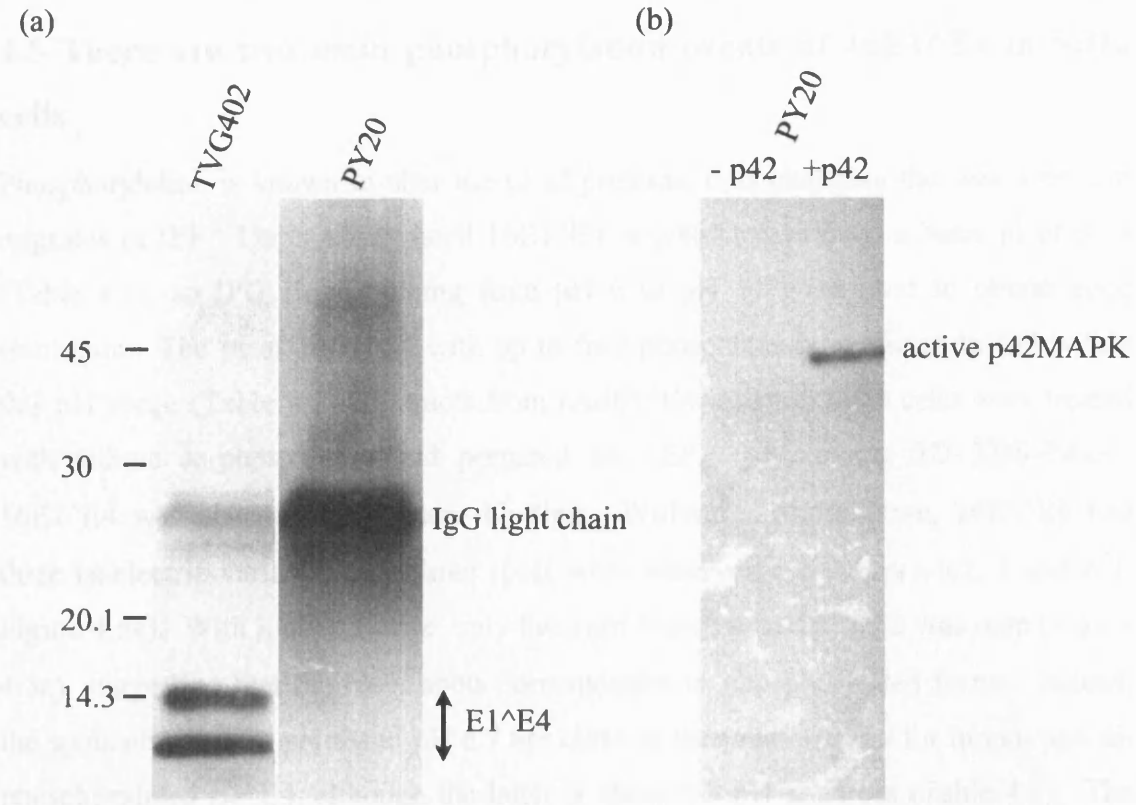


Figure 4.4 Immunoprecipitation of E1^E4 and Western blot with an anti-phosphotyrosine antibody

(a) E1^E4 was expressed in SiHa cells using rAd, then immunoprecipitated using an anti-MBPE1^E4 rabbit polyclonal. Immunoprecipitated E1^E4 was detected by Western blotting using the mouse monoclonal, TVG402. The same membrane was probed with the anti-phosphotyrosine antibody, PY20. (b) Bacterially expressed recombinant p42MAPK containing phosphotyrosine was run in a separate lane and detected by PY20.

4.5 There are two main phosphorylation events of 16E1^E4 in SiHa cells

Phosphorylation is known to alter the pI of proteins, thus changing the way a protein migrates in IEF. Unphosphorylated 16E1^E4 is predicted to have a basic pI of 9.14 (Table 4.1), so IPG strips ranging from pH 6 to pH 11 were used to obtain good resolution. The pI of 16E1^E4 with up to four phosphates is predicted to fall within this pH range (Table 4.1). Extracts from rAdE1^E4-infected SiHa cells were treated with/without λ phosphatase and prepared for IEF. Following 2D SDS-PAGE, 16E1^E4 was detected by Western blotting. Without λ phosphatase, 16E1^E4 had three isoelectric variants since three spots were observed (pI values ~9.2, 8 and 6.7; Figure 4.5a). With λ phosphatase, only the most basic spot of pI ~9.2 was seen (Figure 4.5a), suggesting that the other spots corresponded to phosphorylated forms. Indeed, the spots observed at pH 8 and pH 6.7 are close to the predicted pIs for mono- and di-phosphorylated E1^E4, although the latter is about 0.3 pH units out (Table 4.1). The existence of more than two phosphorylation sites is possible, but these forms (16E1^E4 with three or four phosphates) are predicted to appear within the pH 6-11 range but were not detected by Western blotting with TVG402. Attempts were made to separate the λ phosphatase-negative sample in pH 3-10 IPG strips in order to identify any acidic forms (Figure 4.5b). However, the focusing was very poor and the phosphorylated forms seen with pH 6-11 were now difficult to visualise. IEF of basic proteins is notoriously difficult because of problems with the reducing agent, DTT, migrating towards the acidic end, leaving basic proteins vulnerable to disulphide bonding and desolubilisation (Bae et al., 2003). This may explain the poor focusing in the pH 3-10 gradient compared with the focusing in pH 6-11 gradients.

4.6 Expression of phosphorylation mutants of 16E1^E4 in SiHa cells

To investigate the role of 16E1^E4 phosphorylation, the *in vitro* phosphorylation sites mapped in Chapter 3 were mutated to alanines to produce phosphorylation mutants for expression in SiHa cells. The pMV11.16E1^E4 construct, which contains a CMV promoter, was used as the template to perform site-directed mutagenesis to produce the mutants. A S32A mutant was produced to remove potential CDK1/2 phosphorylation,

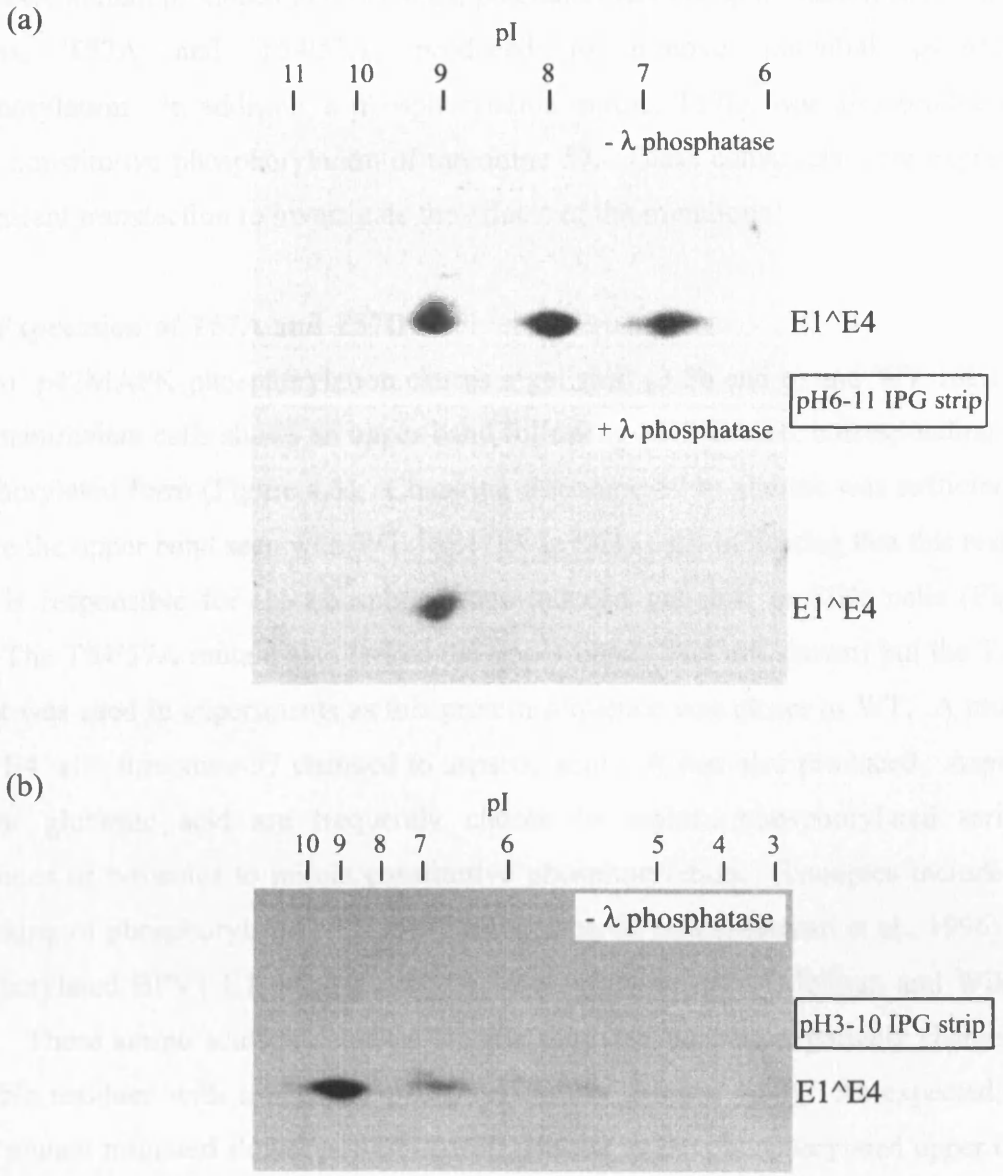


Figure 4.5 2D SDS-PAGE of E1^E4 expressed using rAd
(a) 2D SDS-PAGE of E1^E4 expressed in SiHa cells using rAd. A pH gradient of pH 6-11 was used, and Western blotting with TVG402 performed to detect E1^E4. The cell extracts had been treated with (+) or without (-) λ phosphatase. (b) Western blot to detect E1^E4 from the λ phosphatase-negative sample separated by 2D SDS-PAGE in a pH 3-10 gradient.

a S43/44A mutant produced to remove the potential PKA phosphorylation site, and two mutants, T57A and T54/57A, produced to remove potential p42MAPK phosphorylation. In addition, a phosphorylation mimic, T57D, was also produced to mimic constitutive phosphorylation of threonine 57. These constructs were expressed by transient transfection to investigate the effects of the mutations.

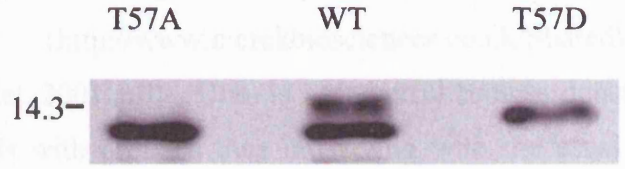
4.6.1 Expression of T57A and T57D

In vitro, p42MAPK phosphorylation causes a gel shift (3.5b and c) and WT 16E1^E4 from mammalian cells shows an upper band following SDS-PAGE, corresponding to a phosphorylated form (Figure 4.3). Changing threonine 57 to alanine was sufficient to remove the upper band seen with WT 16E1^E4 in SiHa cells indicating that this residue alone is responsible for the phosphorylation-induced gel-shift in SiHa cells (Figure 4.6a). The T54/57A mutant also lacked the upper band (data not shown) but the T57A mutant was used in experiments as this protein sequence was closer to WT. A mutant 16E1^E4 with threonine 57 changed to aspartic acid (D) was also produced. Aspartic acid or glutamic acid are frequently chosen to replace phosphorylated serines, threonines or tyrosines to mimic constitutive phosphorylation. Examples include the mimicking of phosphorylated HPV16 E7 using aspartic acid (Massimi et al., 1996) and phosphorylated BPV1 E1 using aspartic acid or glutamic acid (McShan and Wilson, 2000). These amino acids are chosen because they can become negatively charged to resemble residues with a negative phosphate group (Figure 4.6b). As expected, the T57D mutant migrated slower in SDS-PAGE, similar to the phosphorylated upper band of WT 16E1^E4 (Figure 4.6a). This is a good indication that this mutant can mimic phosphorylation.

4.6.2 Solubility fractionation of WT and mutant 16E1^E4

WT and mutant 16E1^E4 were expressed by transfection in SiHa cells. At 24 h post-transfection, the cells were harvested and fractionated. The cells were lysed in 0.5 % NP40 and then centrifuged to obtain the supernatant which was the NP40-soluble fraction. The remaining cell extract (the pellet) was suspended in 0.8 % Empigen to obtain the Empigen-soluble fraction and the residual pellet was suspended in 9 M urea to obtain the insoluble fraction. NP40 is a non-ionic detergent useful for extracting

(a)



(b)

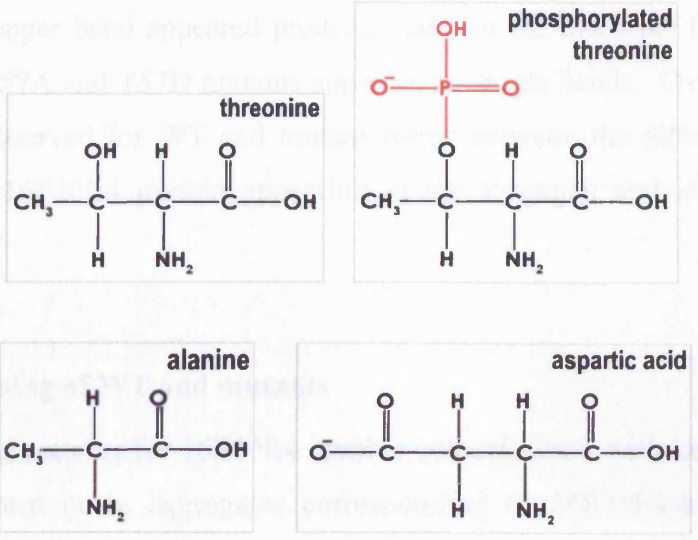


Figure 4.6 Mutating threonine 57 to alanine and aspartic acid
(a) Western blot of T57A, WT and T57D E1^E4 expressed in SiHa cells by transfection. (b) Structures of threonine, phosphorylated threonine and the amino acids used to replace threonine 57, alanine (A) and aspartic acid (D).

membrane proteins, while Empigen is a zwitterionic detergent useful for solubilising keratins. Detergents can solubilise proteins by interaction of the hydrocarbon tail of the detergent with the hydrophobic region of the protein to form a suspension of detergent-protein complexes (http://www.merckbiosciences.co.uk/SharedImages/Technical Literature/1_Detbooklet_2001.pdf). Urea is a powerful protein denaturant that forms strong hydrogen-bonds with proteins thus interfering with the usual hydrogen-bonds between proteins and water molecules (Tobi et al., 2003).

Following fractionation, the 16E1^E4 was detected by Western blotting. The S32A and S43/44A mutants had an upper band and appeared similar to WT (Figure 4.7a). Interestingly, the upper band appeared predominantly in the insoluble (urea) fraction. As expected, the T57A and T57D mutants appeared as single bands. Overall, a similar distribution was observed for WT and mutant forms between the different fractions, with most of the 16E1^E4 protein appearing in the Empigen and insoluble (urea) fractions.

4.6.3 Immunostaining of WT and mutants

The typical staining patterns for 16E1^E4 involve colocalisation with keratin, either in a filamentous pattern or in aggregates corresponding to 16E1^E4-induced keratin collapse. To decipher any variations in cellular distribution resulting from the mutations, the S32A, S43/44A, T57A and T57D mutants together with the WT, were also analysed by fluorescent immunostaining at 24 h post-transfection. 16E1^E4 was detected with the antibody, TGV405, and keratin was detected using an anti-pan-keratin antibody. The staining patterns were divided into four categories: i) keratin and 16E1^E4 colocalisation and no keratin collapse, ii) keratin colocalisation and partial keratin collapse, iii) keratin colocalisation and full keratin collapse or iv) no keratin colocalisation (Figure 4.7b). No keratin colocalisation could mean colocalisation with mitochondria, but mitochondria were not stained in this experiment, or it could mean 16E1^E4 has already caused keratin collapse and the keratin has been degraded (personal communication, Peter Laskey). Low-level 16E1^E4 that was diffuse (i.e. did not form filamentous or collapsed structures) and difficult to detect by immunostaining, was not included in the analysis.

16E1^E4-positive cells were counted and classed into one of the four categories. The experiments were performed in triplicate, using 300 cells in total for each type of 16E1^E4 tested. The means were plotted and the standard deviations indicated (Figure 4.7b). The results showed that the vast majority of cells (about 70 %) had 16E1^E4 and keratin colocalisation without keratin collapse and there were no significant differences in 16E1^E4/keratin patterns between the WT and mutants. Of course, there was likely to have been a sub-population of cells with low-level 16E1^E4 which could not be analysed.

4.7 Identification of 16E1^E4 phosphorylation sites in SiHa cells

The requirement for T57 for the occurrence of the gel-shift is clear, since the T57A mutant lacks the upper band that the WT protein shows. However, whether the absence of the gel-shift with T57A is actually due to lack of phosphorylation or a structural effect of alanine replacing threonine, needed to be determined. It was also necessary to investigate whether S32 or S43/44 are actually phosphorylated in SiHa cells. It was decided to analyse each mutant by 2D SDS-PAGE to compare the number of phosphorylated forms to that of WT. The WT and mutant proteins were expressed by transfection of pMV11 constructs. When WT 16E1^E4 was separated by 2D SDS-PAGE and Western blotted, only the spots at pH 9.2 and 8 were easily seen and a third spot at pH 6.7 observed only after overnight exposure (Figure 4.8a). This is different to the rAd-expressed 16E1^E4 that gave three clear spots, suggesting that rAd infection may enhance the level of phosphorylation.

In order to compare phosphorylation of the mutants with the WT, however, conditions permitting optimal phosphorylation levels were required. WT 16E1^E4 was expressed by transfection, the cells incubated with the phosphatase inhibitor, OA, for one hour before harvest (cells were harvested at 24 h post-transfection). OA was also added during lysis of the cells. 2D SDS-PAGE and Western blotting were performed to detect the WT 16E1^E4, and this time, spots at ~pH 8 and ~pH 6.7 were seen, corresponding to mono- and di-phosphorylated 16E1^E4 respectively (Figure 4.8b). OA treatment therefore resulted in complete 16E1^E4 phosphorylation as the

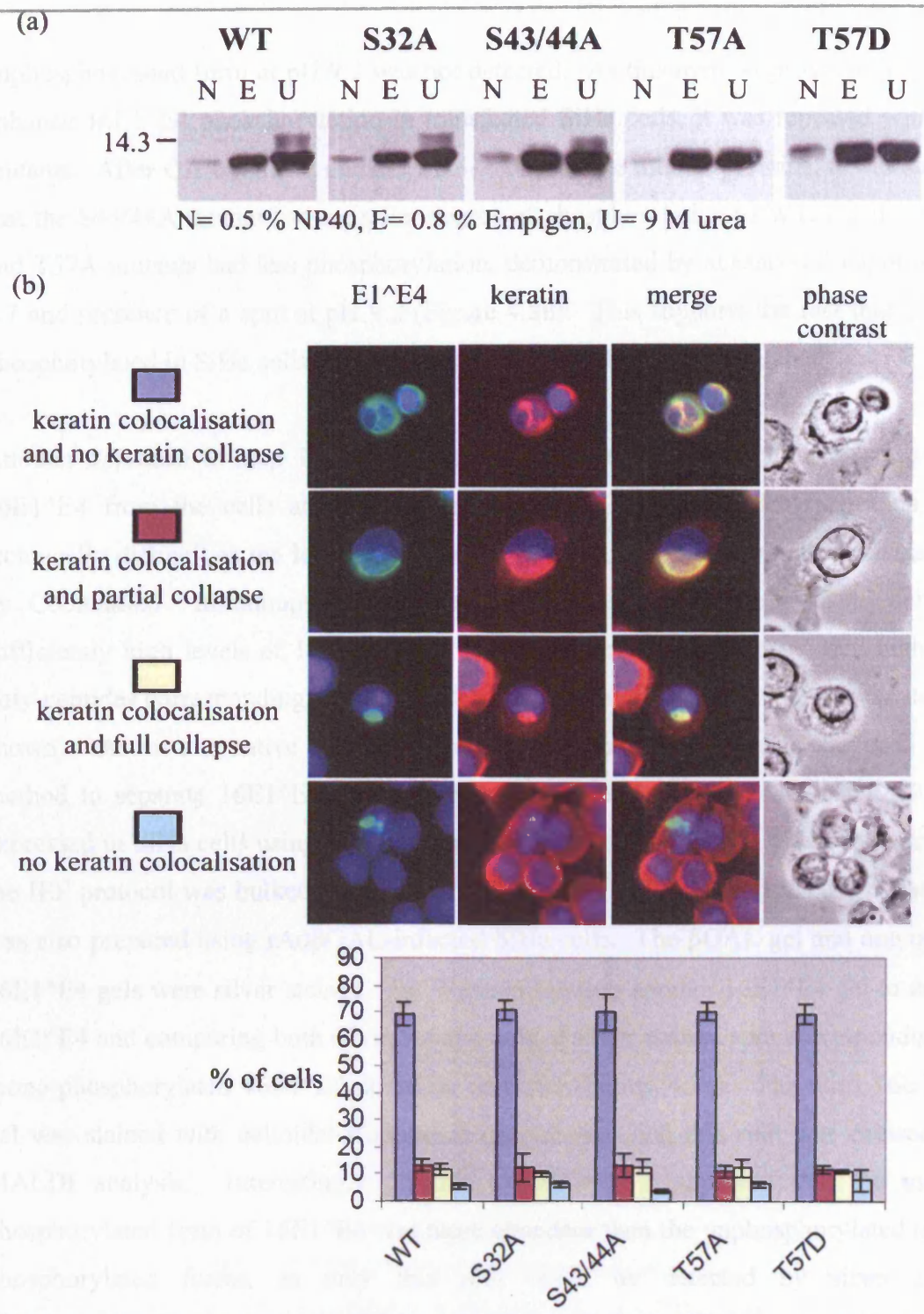


Figure 4.7 Fractionation and immunostaining of phosphorylation mutants
(a) Western blots using TVG402 to detect WT and mutant E1^E4s following solubility fractionation using NP40, Empigen and urea solutions. (b) SiHa cells were immunostained for E1^E4 and keratin at 24 h post-transfection of WT and mutant E1^E4 constructs. The staining patterns were classed into four categories: 'keratin and E1^E4 colocalisation and no keratin collapse', 'keratin colocalisation and partial collapse', 'keratin colocalisation and full collapse' or 'no keratin colocalisation'. An example of each category is indicated showing E1^E4 in green and keratin in red. The percentage of cells in each category were recorded and mean values represented in a bar graph. The error bars indicate the standard deviation.

unphosphorylated form at pH 9.2 was not detected. As this method provided a way to enhance 16E1^E4 phosphorylation in transfected SiHa cells, it was repeated with the mutants. After OA treatment and 2D SDS-PAGE of the mutant proteins, it was shown that the S43/44A mutant had a similar degree of phosphorylation to WT, but the S32A and T57A mutants had less phosphorylation, demonstrated by absence of a spot at pH 6.7 and presence of a spot at pH 9.2 (Figure 4.8b). This supports the fact that T57 is phosphorylated in SiHa cells and suggests that S32 is also phosphorylated.

Another approach to map 16E1^E4 phosphorylation sites in cell culture is to purify 16E1^E4 from the cells and analyse it by MS. This is the ideal approach, but technically difficult as the level of protein required must be high enough to be stained by Coomassie. Immunoprecipitation of 16E1^E4 was attempted but to achieve sufficiently high levels of 16E1^E4, the amount of antibody used was very high and only peptides corresponding to antibody fragments were detected by MALDI (data not shown). As an alternative to immunoprecipitation, an IEF approach was used as a method to separate 16E1^E4 from cellular proteins and use for MS. 16E1^E4 was expressed in SiHa cells using rAdE1^E4 and separated by SDS-PAGE in triplicate, but the IEF protocol was bulked-up ten-fold for each IEF run. A negative control sample was also prepared using rAdβGAL-infected SiHa cells. The βGAL gel and one of the 16E1^E4 gels were silver stained. By Western blotting another 16E1^E4 gel to detect 16E1^E4 and comparing both silver stained gels, a silver stained spot corresponding to mono-phosphorylated 16E1^E4 could be detected (Figure 4.9a). The third 16E1^E4 gel was stained with colloidal Coomassie (not shown) and this spot was excised for MALDI analysis. Interestingly, in this experiment, it appeared that the mono-phosphorylated form of 16E1^E4 was more abundant than the unphosphorylated or di-phosphorylated forms, as only this spot could be detected by silver stain. Chymotrypsin digestion and MALDI revealed an ion of m/z 2066 corresponding to the unphosphorylated, oxidated (+ $2O_2$) peptide GSTWPTTPPRPIPKPSPW (amino acids 17-34) and an ion of m/z 2868 corresponding to the presence of one phosphate in the peptide APKKHRRLSSDQDQSQTPETPATPL (amino acids 35-59; Figure 4.9b). Exact mapping by collision-induced fragmentation was not possible as the protein level was too low. However, the MALDI result does support the existence of phosphorylation at T57 and suggests that S32 phosphorylation does not occur in the mono-phosphorylated form. It is tempting to speculate that T57 phosphorylation may

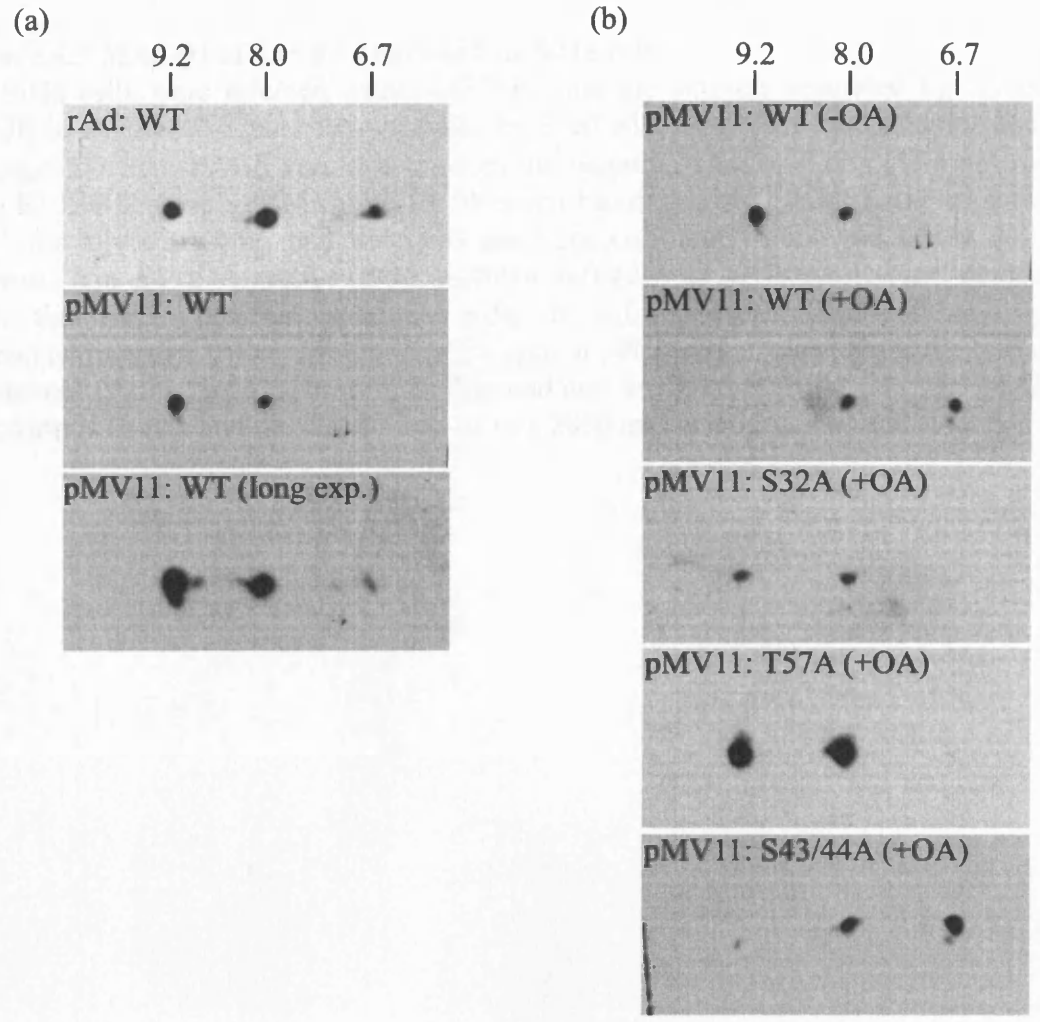
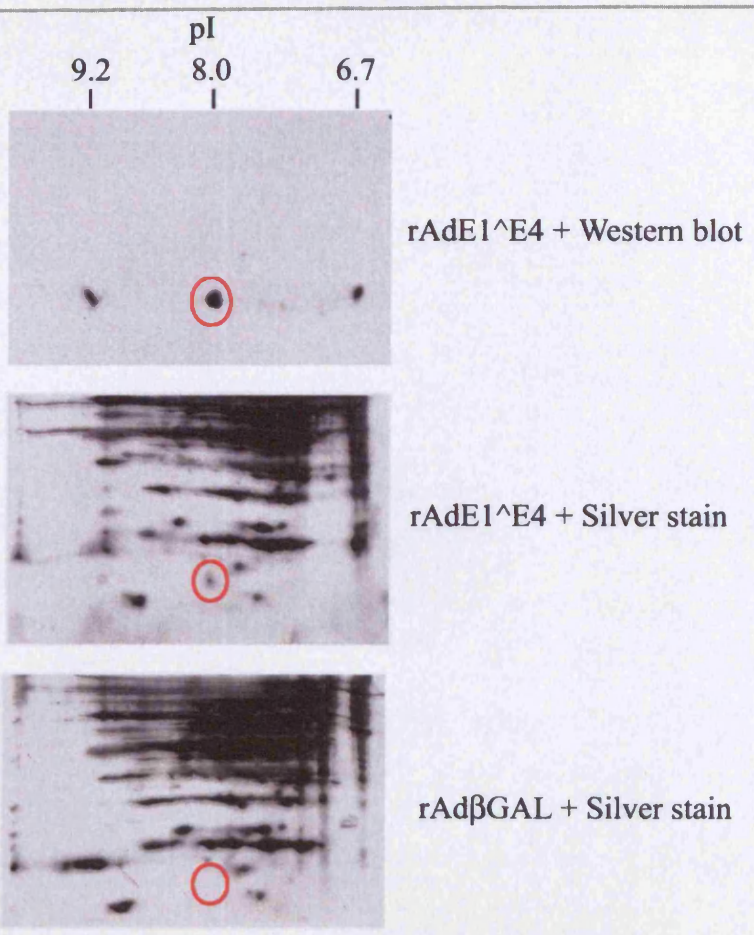


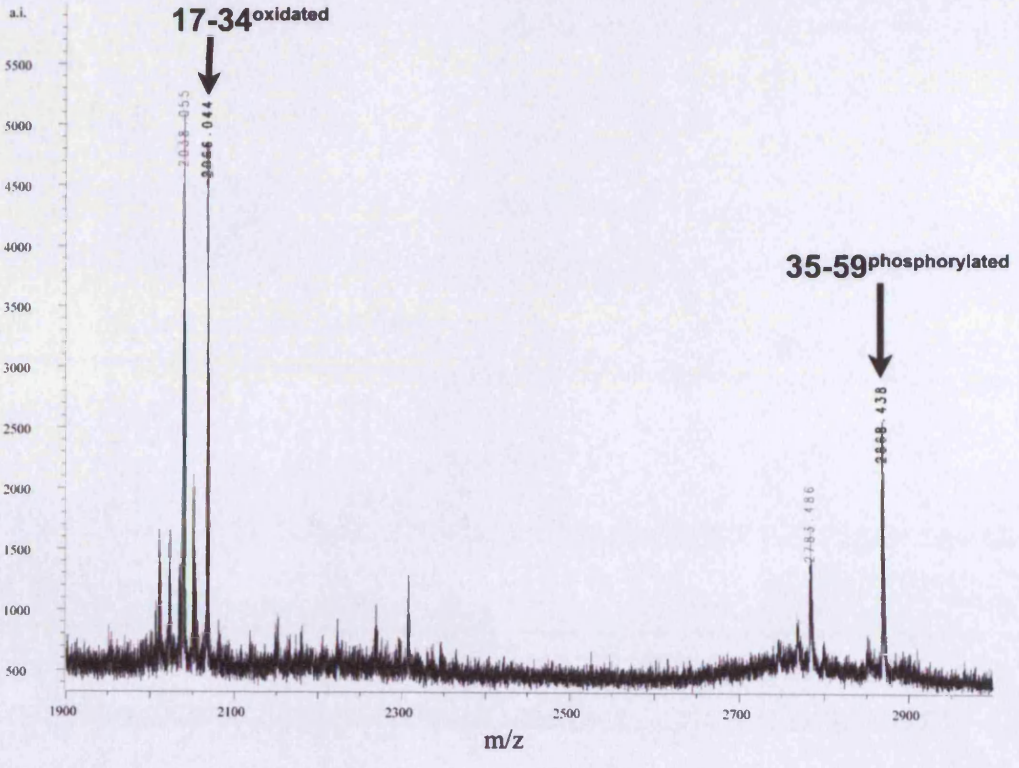
Figure 4.8 2D SDS-PAGE of phosphorylation mutants
(a) WT E1^E4 expressed by transfection (with a pMV11 construct) and WT E1^E4 expressed using rAd, were compared by 2D SDS-PAGE. Western blotting with TVG402 was used to detect E1^E4. An overnight exposure of the Western blot of the transfection sample, is also shown. (b) E1^E4 was expressed by transfection and WT E1^E4 treated with (+) or without (-) OA. Mutant E1^E4 samples were treated with OA. All samples were compared by 2D SDS-PAGE and Western blotting.

Figure 4.9 MALDI of E1^E4 expressed in SiHa cells
(a) SiHa cells were infected with rAdE1^E4 and the extracts separated by 2D SDS-PAGE in triplicate. SiHa cells were also infected with rAdβGAL, the sample used for a single 2D SDS-PAGE run, and used as the negative control (i.e. E1^E4-negative). One E1^E4-positive gel was used for Western blotting with TVG402, the second gel used for silver staining and the third used for colloidal Coomassie (third gel not shown). The E1^E4-negative (βGAL-containing) gel was silver stained and compared with the E1^E4-positive gels, in order to allow identification of any spots corresponding to E1^E4. (b) The E1^E4 spot at pH 8 was excised from the colloidal Coomassie-stained gel, chymotrypsin digested and analysed by MALDI. The MALDI spectrum is shown and the E1^E4 ions of m/z 2066 and m/z 2868 are indicated.

(a)


pI
9.2
8.0
6.7
rAdE1^E4 + Western blot
rAdE1^E4 + Silver stain
rAdβGAL + Silver stain

(b)


a.i.
17-34oxidated
35-59phosphorylated
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
1900
2100
2300
2500
2700
2900
m/z

occur before S32 phosphorylation.

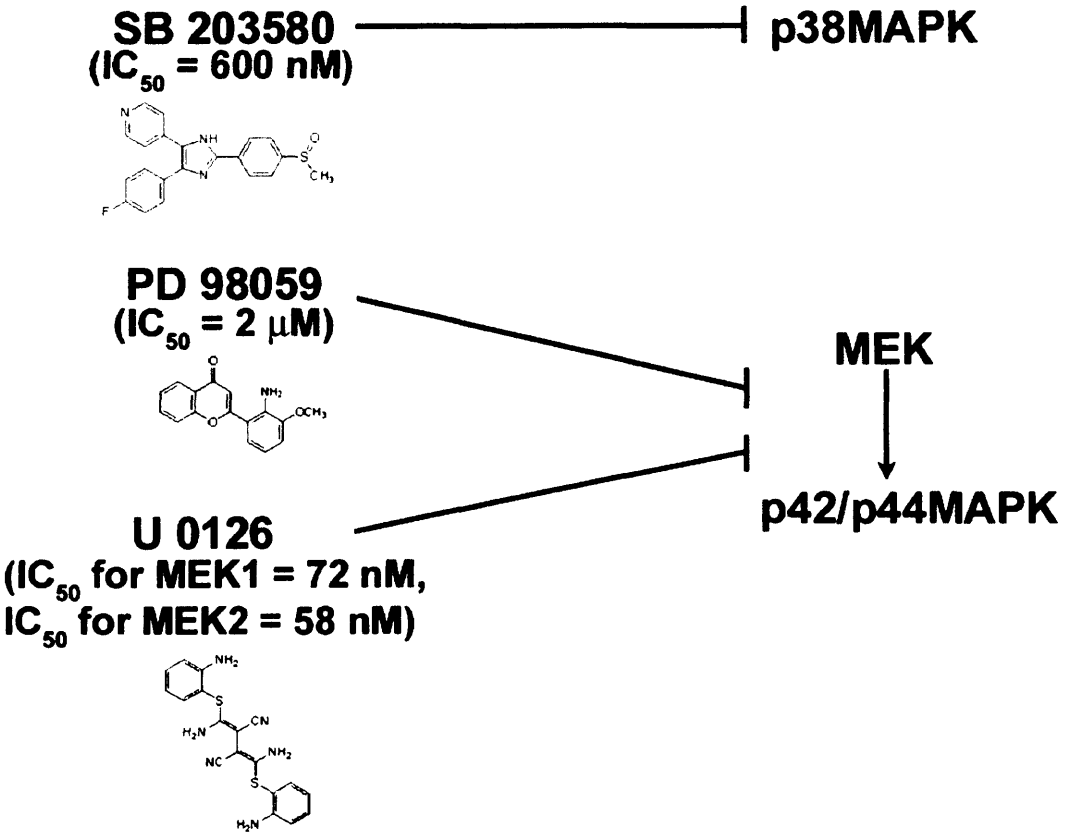
4.8 MEK inhibitors reduce 16E1^E4 phosphorylation in SiHa cells

Kinase inhibitors were used to find whether a MAPK pathway is involved in the phosphorylation of 16E1^E4 in SiHa cells. The inhibitors, SB203580, PD98059 and U0126 or DMSO (negative control) were added to the cell culture media 6 h following infection of the SiHa cells with rAdE1^E4. The cells were harvested after incubation with the inhibitors for 18 h. SB203580 inhibits p38MAPK by blocking its ATP binding site and PD98059 and U0126 bind MEK1 and MEK2 in a non-ATP competitive manner preventing them from activating p42 or p44MAPK (English and Cobb, 2002; Figure 4.10a). After the cell extracts were analysed by Western blotting to detect 16E1^E4, it was seen that the upper band was reduced in the samples treated with MEK inhibitors but not in the sample treated with the p38 inhibitor or in the negative control (Figure 4.10b). This suggests that downstream effectors of MEK cause the phosphorylation-induced gel-shift of 16E1^E4. MEK activates p42 and p44MAPK (Figure 1.4) so one or both of these MAPKs are likely to phosphorylate 16E1^E4 to produce the upper band.

4.9 A CDK1/2 inhibitor reduces 16E1^E4 phosphorylation in SiHa cells

To test for a role for CDK1 or CDK2 in 16E1^E4 phosphorylation, the CDK1/2 inhibitor, Roscovitine, was used (Figure 4.11a). Roscovitine inhibits CDK1/2 by competing with ATP but does not inhibit CDK4/cyclin D or CDK6/cyclin D complexes (Meijer et al., 1997). SiHa cells were infected with rAdE1^E4 for 6 h, then Roscovitine or DMSO (as a negative control) was added to the cell culture media. The cells were harvested 16 h later and the extracts subjected to 2D SDS-PAGE. Following Western blotting with TVG402, it was seen that the Roscovitine-treated sample showed a significantly weaker spot at pH 6.7 compared with the Roscovitine-negative sample (Figure 4.11b). The spots at pH 9.2 and 8 were similar in both samples. The fact that the presence of Roscovitine lowered the degree of phosphorylation suggests that CDK1

(a)



(b)

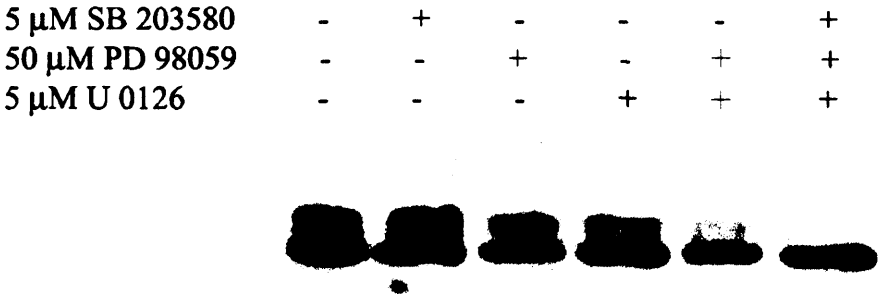
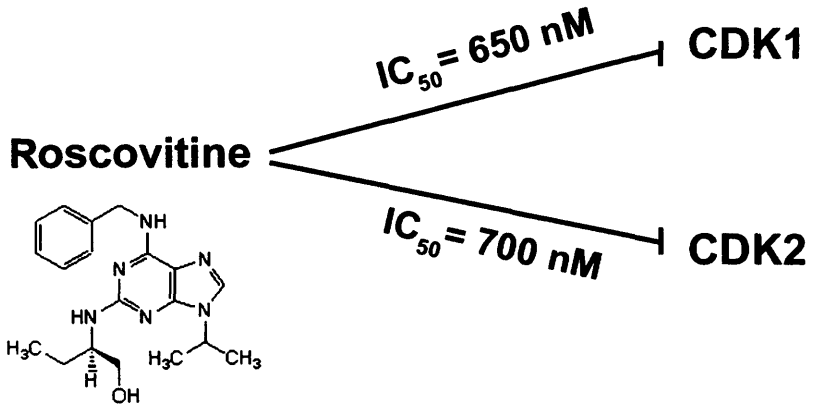


Figure 4.10 Treatment with MAPK inhibitors

(a) The structures of the MAPK inhibitors, SB 203580, PD 98059 and U 0126 are illustrated and their IC_{50} values indicated (IC_{50} is the concentration of inhibitor needed to inhibit 50 % of the reaction). (b) Western blot of E1^E4 expressed (using rAd) in SiHa cells, which were incubated with the different MAPK inhibitors. Each inhibitor was used alone and in conjunction with others.

(a)



(b)

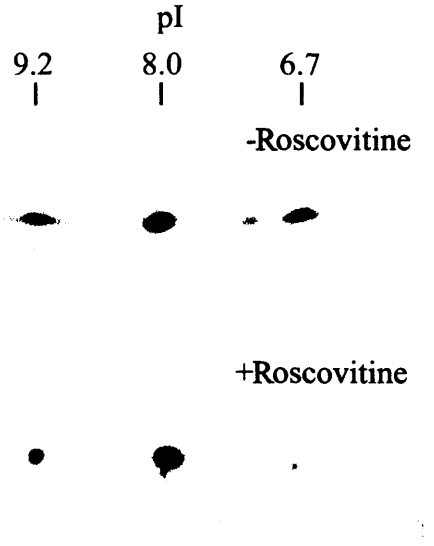


Figure 4.11 Treatment with Roscovitine
(a) The structure of the CDK1 and CDK2 inhibitor, Roscovitine and its IC_{50} value are shown. (b) Western blots of E1^E4 expressed using rAd in SiHa cells and separated by 2D SDS-PAGE (in pH 6-11). The rAd-infected cells had been treated with (+) or without (-) 30 μM Roscovitine.

or CDK2 or both, can cause phosphorylation of 16E1^E4 in SiHa cells.

4.10 Discussion

4.10.1 16E1^E4 phosphorylation events in cell culture

16E1^E4 was expressed in cultured cells to reveal different migratory forms following SDS-PAGE. In all cells tested, a 14 kDa band and 13 kDa band were seen and in C33A cells, a 10 kDa form was detected. The upper band (14 kDa) was shown to correspond to a phosphorylated form while the 10 kDa band was an N-terminally truncated form. A phosphorylated form of 16E1^E4 that causes a gel-shift has not been previously reported in any detail, but N-terminally truncated forms of E1^E4 have been reported particularly for HPV1.

The phosphorylation status of 16E1^E4 expressed in SiHa cells was analysed to reveal that threonine 57 and serine 32 are phosphorylated. Threonine 57 phosphorylation is mediated by the MEK-p42/p44MAPK pathway and does not appear to involve p38MAPK. This phosphorylation event appears to cause a structural change since it causes a slower-migrating band in SDS-PAGE. Serine 32 is phosphorylated by CDK1/cyclin or CDK2/cyclin or both. Phosphorylation of tyrosine was not detected with an anti-phosphotyrosine antibody. Similarly, phosphoamino acid analysis of 11E1^E4 from xenograft tissue did not detect phosphotyrosine (Bryan et al., 2000).

The evidence for these sites being phosphorylated comes from IEF experiments which suggest that phosphorylation is reduced in the T57A and S32A mutants. In addition, the T57A mutant loses the slower-migrating, phosphorylation-induced form that the WT possesses, whereas the T57D phosphorylation mimic appears as a slower-migrating band. Also, when WT 16E1^E4 was separated by IEF and analysed by MALDI, the mono-phosphorylated form was found to possess a phosphate within amino acids 35 to 59. Interestingly, no phosphorylation at serine 32 was detected by MALDI in this mono-phosphorylated form. This suggests that in rAd-infected SiHa cells, threonine 57 phosphorylation may take place before serine 32 phosphorylation. This may occur simply because the opportunity for threonine 57 phosphorylation is greater, for example if MAPK activity is particularly high in SiHa cells. Alternatively,

serine 32 phosphorylation could be dependent on threonine 57 phosphorylation due to a change in protein conformation or location.

There is the possibility, however, that these sites are not normally phosphorylated, but mutating them alters the protein structure in such a way as to affect normal phosphorylation. However, the fact that *in vitro* phosphorylation data also involves these residues adds strong support to the prediction that these sites are phosphorylation sites *in vivo*.

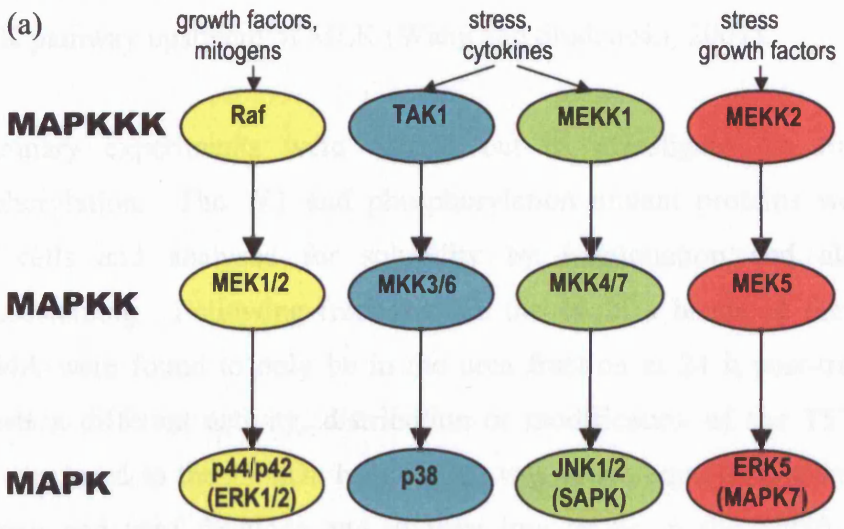
There is no evidence for more than two phosphorylation events for 16E1^E4 in SiHa cells because only mono- and di-phosphorylated forms were detected by IEF, but other phosphorylation events may occur that are either minor events or not detected by the TGV402 antibody (see Table 2.8 for epitopes recognised). There may also be phosphorylation events that are cell cycle-dependent so may be undetected at the timepoint used in this study (24 h post-transfection or post-rAd infection). When the *in vitro* PKA site (serine 43) was mutated for expression in SiHa cells, the IEF profile indicated the same number of phosphorylation events as WT, suggesting that this site is not phosphorylated in SiHa cells. This may not be the case, however, as S43 could be phosphorylated in a cell cycle-dependent manner. In this experiment the mutant used was actually a S43/44A mutant since serine 44 is phosphorylated by PKA *in vitro* in the absence of serine 43. This S43/44A mutant may have a structural form that permits phosphorylation at a residue that is not normally phosphorylated in the WT thus masking the lack of S43 phosphorylation. Even if S43 is not phosphorylated in cultured SiHa cells, the possibility that it is an important functional target of kinases in HPV-infected epithelia should be considered in future projects. As well as phosphorylation by PKA, phosphorylation by PKC could occur *in vivo* especially considering the importance of PKC in epithelial growth and differentiation (Denning, 2004; Yang et al., 2003).

The use of MEK inhibitors implicated p42 or p44 MAPK in 16E1^E4 phosphorylation in SiHa cells. Interestingly, the p42/p44MAPK pathway has been shown to be activated by high-risk HPV E6 proteins, including 16E6 (Chakrabarti et al., 2004), and SiHa cells express 16E6. HPV11 E1^E4 has also been shown to be phosphorylated by MAPK *in vitro* at threonine 53 and mass spectrometry data supported the

phosphorylation of this site *in vivo* (Bryan et al., 2000). The p42 and p44 MAPKs are widely expressed and involved in the regulation of meiosis and mitosis and also have postmitotic functions in differentiated cells (Johnson and Lapadat, 2002). As well as phosphorylating nuclear transcription factors (Figure 1.4), p42/p44MAPK can phosphorylate other proteins such as the kinase, p90rsk/MAPKAP-K1 (Dalby et al., 1998) and the microtubule-associated protein, tau (Illenberger et al., 1998). Although inhibiting p38MAPK did not appear to affect 16E1^E4 phosphorylation, other MAPK pathways (Figure 4.12) such as the jun N-terminal protein kinase (JNK) pathway (also known as stress-activated protein kinase/SAPK) may be involved and could be investigated in future experiments.

The use of Roscovitine implicated CDK1/2 in 16E1^E4 phosphorylation. CDK1 can associate with cyclin A or cyclin B and CDK2 can bind cyclin A or cyclin E and these complexes act to regulate cell cycle progression (Figure 4.12). CDK/cyclin A complexes control DNA replication, centrosome duplication and play a role in mitotic entry, and active CDK1/cyclin B is required for mitosis. Phosphorylation substrates of CDK/cyclin A include pRB which becomes further inactivated (Zarkowska and Mittnacht, 1997) and the transcription factor, upstream binding factor (UBF) which becomes activated and increases transcription of rRNA (Voit and Grummt, 2001). The targets of CDK1/cyclin B include the nuclear lamins, which become depolymerised leading to nuclear envelope breakdown in mitosis (Peter et al., 1990). The finding that CDK can phosphorylate 16E1^E4 is significant given that CDK1/cyclin B (Davy et al., 2005) and CDK2/cyclin A (Davy et al., 2006) can bind 16E1^E4 in cells. CDK1/cyclin B binding is believed to be the mechanism for 16E1^E4-mediated G_2 arrest, caused by 16E1^E4 sequestering CDK1 from its normal substrates. The significance of CDK2/cyclin A association is uncertain, however, it is likely to affect normal CDK2/cyclin A activity. It would be interesting to test whether the S32A mutant, which is no longer phosphorylated *in vitro* by CDK1/cyclin B, retains the ability to bind cyclin B and cause G_2 arrest.

Although the use of the inhibitors implicated these kinases in 16E1^E4 phosphorylation, pharmacological inhibitors may have pleiotropic effects and not just block the kinase of interest. For example, the MEK inhibitors, PD98059 and U0126, can activate AMP-activated protein kinase (AMPK; Dokladda et al., 2005) and can



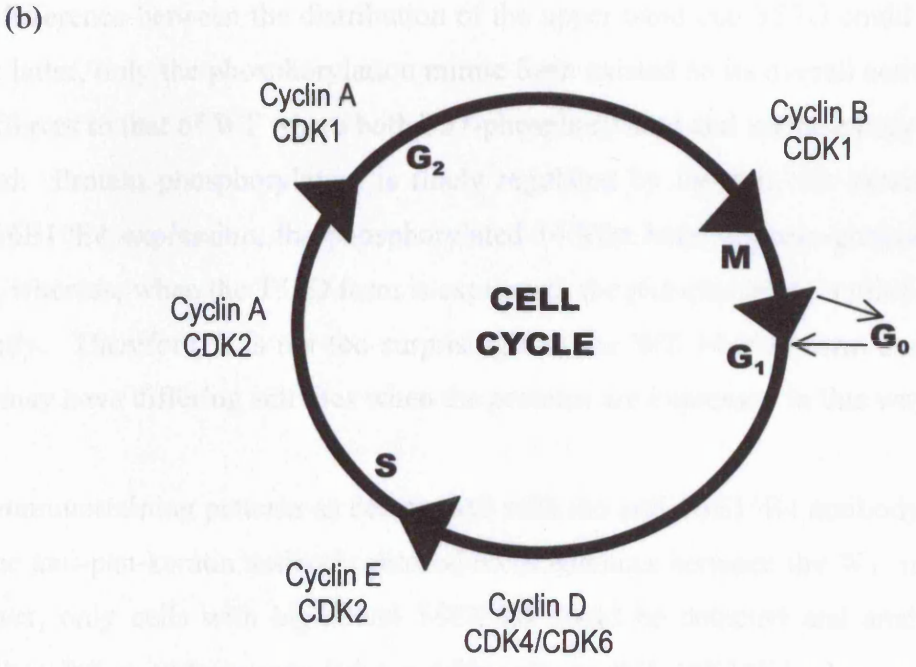


Figure 4.12 Outline of the MAPK pathways and cell cycle roles of CDK/cyclin complexes
(a) Diagram of the main MAPK pathways. The p42/p44MAPK is involved in 16E1^E4 phosphorylation. (b) The stages of the cell cycle and associated CDK/cyclin complexes are indicated. 16E1^E4 can associate with and be phosphorylated by CDK2/cyclinA and CDK1/cyclin B.

inhibit kinase suppressor of ras (KSR-1), a kinase that can activate raf-1, so affect the MAPK pathway upstream of MEK (Wang and Studzinski, 2001).

Preliminary experiments were carried out to investigate the role of 16E1^E4 phosphorylation. The WT and phosphorylation mutant proteins were expressed in SiHa cells and analysed for solubility by fractionation and also analysed by immunostaining. Following fractionation, the 14 kDa bands of the WT, S32A and S43/44A were found to only be in the urea fraction at 24 h post-transfection. This suggests a different activity, distribution or modification of the T57-phosphorylated form compared to the 13 kDa band which was found equally distributed between the Empigen and urea fractions and at very low levels in the NP40-soluble fraction. However, the phosphorylation mimic, T57D, also appeared at equal levels in the Empigen and urea fractions and at very low levels in the NP40 fraction.

This difference between the distribution of the upper band and T57D could be because in the latter, only the phosphorylation mimic form existed so its overall activities could be different to that of WT where both T57-phosphorylated and unphosphorylated forms existed. Protein phosphorylation is finely regulated by the cell, for example, during WT 16E1^E4 expression, the phosphorylated 14 kDa band appears gradually (Figure 4.2c), whereas, when the T57D form is expressed, the phosphorylation mimic is present instantly. Therefore, it is not too surprising that the WT 14 kDa form and the T57D form may have differing activites when the proteins are expressed in this way.

The immunostaining patterns as determined with the anti-16E1^E4 antibody, TGV405, and the anti-pan-keratin antibody showed no differences between the WT and mutants, however, only cells with high-level 16E1^E4 could be detected and analysed. The phosphorylation mutants may behave differently to WT 16E1^E4 when protein levels are lower and below saturation. Differences may be more apparent in a system more similar to the *in vivo* situation, for example if the proteins are expressed from the HPV genome.

4.10.2 Abundance of 16E1^E4 phosphorylation in cell culture

The presence of di-phosphorylated 16E1^E4 in SiHa cells appears to be greater in rAd-

infected cells compared to transfected cells. In this study, the di-phosphorylated form (IEF spot at pH 6.7) level could be greatly increased using okadaic acid treatment of transfected cells, without which, this form was difficult to detect. The difference in phosphorylation observed between transfected and infected cells could be due to transfection causing a decrease in cellular phosphorylation or adenovirus infection causing an increase in cellular phosphorylation or both. For example, Hela cells (HPV18-transformed cervical cancer cell line) have been shown to have altered cell cycling and increased apoptosis following transient transfection (Rodriguez and Flemington, 1999). It is therefore possible that these effects may also occur with transfected SiHa cells, thus affecting cell signalling and phosphorylation. In the adenovirus-infected cells, it is likely that the activity of the adenovirus proteins contributed to the increased phosphorylation, in particular, the activity of the adenovirus E4 ORFs.

The replication-defective adenoviruses used in this study lack the adenovirus E1 and E3 ORFs but retain E4 ORFs (see Appendix.4 for vector map). The E4 ORFs are known to have effects on transcription and cell signalling, and this may have implications for the use of adenoviruses as vectors (Weitzman, 2005). For example, E4ORF4 interacts with and alters the substrate specificity of the c-src tyrosine kinase and E4ORF6/7 binds to and increases activity of the E2F transcription factor (Weitzman, 2005). E2F-responsive genes include cyclins E and A, so adenovirus E4 could increase HPV16 E1^E4 phosphorylation by CDK/cyclin. In a study where cellular genes induced by an adenovirus vector (lacking E1 but containing E4) were identified, one of the genes was found to be MEK5 (Ramalingam et al., 1999). MEK5 is part of one of the MAPK pathways and can be activated by oxidative stress, mitogens or growth factors (Figure 4.12). It is upstream of ERK5 (also called MAPK7) and the MEK5/ERK5 pathway is essential for cardiac and vascular development and cell survival (Wang et al., 2005; Yan et al., 2003). Given that adenoviruses can affect this MAPK pathway, it is also possible that they may indirectly or directly affect p42/p44MAPK activity although there have been no such reports to date.

After okadaic acid treatment and 2D SDS-PAGE of 16E1^E4 expressed by transfection, the unphosphorylated form of 16E1^E4 was not detected, suggesting that the rate of phosphorylation is high and cellular phosphatases do lower 16E1^E4

phosphorylation. Also, the finding that the mono-phosphorylated form of 16E1^E4 was more abundant than the unphosphorylated form at 24 h post-rAd infection, indicates that most of the 16E1^E4 in cells (at least at that timepoint) is phosphorylated.

MALDI analysis suggested that the mono-phosphorylated form is the T57-phosphorylated form. When 16E1^E4 is detected by Western blotting following SDS-PAGE, the abundance of the upper band is at a maximum of 50 % of the total 16E1^E4. The upper band is a result of T57 phosphorylation, so it was surprising that IEF found that the T57-phosphorylated form was more abundant than unphosphorylated 16E1^E4. An explanation could be that the T57 phosphorylation level is in fact high at 24 h, but the structural change that causes the slow-migration is partially lost during SDS-PAGE. It has been noticed that the upper band is reduced by freeze-thawing of the sample, so although the structural change is apparent by SDS-PAGE, it is somewhat sensitive to denaturation. A slower-migrating spot corresponding to the upper band is not visualised by IEF and this is likely to be because the protein is in denaturing conditions in the presence of urea and thiourea and is in a state where it has no overall charge, therefore charge-specific intra-molecular interactions may be disrupted.

Phosphorylation of 16E1^E4 does indeed appear to be abundant in cells so is likely to play a role in the virus life cycle. In addition to further work with phosphorylation mutants, an ideal method for detecting phosphorylated forms and finding phosphorylation effects in cell culture or *in vivo* is to use phospho-specific antibodies. The production of anti-16E1^E4 phospho-specific antibodies is in progression (see Appendix.5) and will be valuable when complete.

Chapter 5: Further Analysis of 16E1^E4 Threonine 57 Phosphorylation

5.1 Introduction

The phosphorylation of 16E1^E4 at theonine 57 by MAPK was chosen to be further analysed because of its ability to induce a gel-shift that is likely to be due to a structural change. The effect of E5 on MAPK and threonine 57 phosphorylation was also studied. In, addition, the presence of homologous MAPK sites in other E1^E4 proteins, was investigated.

Gel-shifts caused by phosphorylation are a common phenomenon; examples of other proteins that have phosphorylation-induced gel-shifts include pRB (DeCaprio et al., 1989), PKC (Borner et al., 1989), the cardiac muscle protein, phospholamban (Li et al., 1998), and adenovirus E1A (Smith et al., 1989). Most cases are likely to represent structural changes caused by an increased radius (stokes radius) of the polypeptide, as demonstrated for phospholamban by Li et al. (1998). In the case of 16E1^E4, biophysical studies (for example, intrinsic tryptophan fluorescence measurements) have in fact demonstrated that threonine 57 phosphorylation does induce a structural change (personal communication, Dr Pauline McIntosh, NIMR).

An alteration of protein structure is very likely to affect protein activity. It has already been shown by detergent fractionation that the T57-phosphorylated form (the upper band) is present in the insoluble fraction of the cell (Figure 4.7). Phosphorylation-induced insolubility may arise from the structural change per se, oligomerisation, a change in cellular location or association with other proteins to form an insoluble complex. Alternatively, it is possible that T57 phosphorylation occurs specifically in an insoluble cellular compartment such as on keratins. There is in fact, increasing evidence that intermediate filaments act as scaffolds for signalling pathways and several kinases are known to bind them, including, PKC, PKB and c-jun N-terminal kinase (JNK; Coulombe and Wong, 2004).

It is known that 16E1^E4 binds keratin in cell culture and colocalises with keratin *in vivo*. Keratin is mostly an insoluble protein, although during mitosis it is phosphorylated and solubilised. In cultured HT29 cells (colon cancer cells), the distribution of keratins 8 and 18 is as follows; 5 % is soluble in detergent-free buffer, 10 % is NP40-soluble, 45 % is Empigen-soluble and 40 % is Empigen-insoluble (Omary et al., 1998). This distribution of keratin between the Empigen-soluble and insoluble fractions is similar to the distribution of 16E1^E4 but the upper band of 16E1^E4 is primarily in the insoluble fraction. It was hypothesised that T57 phosphorylation may lead to 16E1^E4 having a greater propensity to associate with the insoluble keratins. The effect of T57 phosphorylation on keratin binding was therefore investigated in an *in vitro* assay using keratins 8 and 18 from SiHa cells and bacterially expressed His-E1^E4, both WT and the phosphorylation mimic, T57D. Keratins 8 and 18 are typically expressed in simple epithelia (for example, the gastrointestinal tract and liver) and not in keratinocytes (Omary et al., 1998), however, they are present in the SiHa cancer cell line. 16E1^E4 has been previously shown to colocalise with and directly bind keratin 8 and 18, although the direct binding to keratin 8 was considerably weaker (Doorbar et al., 1991; Wang et al., 2004).

The order of 16E1^E4 phosphorylation events may prove to be significant. For example, phosphorylation mapping data in Chapter 4 suggested that in SiHa cells, threonine 57 phosphorylation occurs before serine 32 phosphorylation, since phosphorylated serine 32 was not detected in the mono-phosphorylated form. This could occur due to less CDK1/2 activity compared to MAPK activity. More interestingly, the reason could be that threonine 57 phosphorylation by MAPK improves the rate at which 16E1^E4 is phosphorylated by CDK1/2. To investigate this, CDK2/cyclin A kinase assays were performed using the T57A and T57D His-E1^E4 mutants. Also, it is known that 16E1^E4 can colocalise with cyclin A/B (Davy et al., 2006; Davy et al., 2005), so the potential of T57A to colocalise with cyclin (in this case, cyclin A) was analysed.

In a HPV16 infection, E1^E4 is expressed alongside other viral proteins (Doorbar, 2005), so its activities should be considered in the context of the viral life cycle. *In vivo,* transcripts that encode both 16E1^E4 and 16E5 are detected (Doorbar et al., 1990; Vormwald-Dogan et al., 1992), so it is likely that these two proteins are co-expressed at

some stage of the viral life cycle. Since 16E5 is known to increase MAPK signalling, its ability to increase 16E1^E4 phosphorylation was tested. The effects of 16E5-induced MAPK signalling may contribute viral replication and virus production and this may partly be through regulation of viral proteins such as 16E1^E4.

5.2 T57D binds keratin more strongly than WT does

To test the binding of 16E1^E4 to keratin and the effect of T57 phosphorylation, keratins were immunoprecipitated from cultured cells and incubated with WT His-E1^E4 or the phosphorylation mimic, T57D His-E1^E4. Keratins 8 and 18 were immunoprecipitated from SiHa cells using the monoclonal mouse antibody, L2A1, and protein G-sepharose beads. The keratin-bound beads were washed and 0.4 μg of WT or T57D were added to the beads. After two hours, the beads were washed, then analysed by SDS-PAGE and Western blotting with the anti-E1^E4 antibody, TVG402.

The results show that both His-E1^E4 proteins could associate with the immunoprecipitated keratins, but the T57D mutant could bind with greater affinity (Figure 5.1). This was repeated twice more (data not shown) and each time, the T57D bound more strongly than WT to the keratin-bound beads.

5.3 Immunostaining of keratin and 16E1^E4 in SiHa cells

The observation that the T57D phosphorylation mimic bound keratin more strongly than the WT protein in the *in vitro* assay (see 5.2), prompted the comparison of the colocalisation of T57A, T57D and WT with keratin in cells. However, when T57A, T57D and WT 16E1^E4 were transfected into SiHa cells and immunostained at 24 h post-transfection, no differences in the degree of keratin-16E1^E4 colocalisation or 16E1^E4-mediated keratin collapse were detected (see 4.6.3). It was therefore decided to test later timepoints to see whether, with time, the T57D or WT protein would show increased keratin colocalisation or collapse in comparison to the T57A mutant.

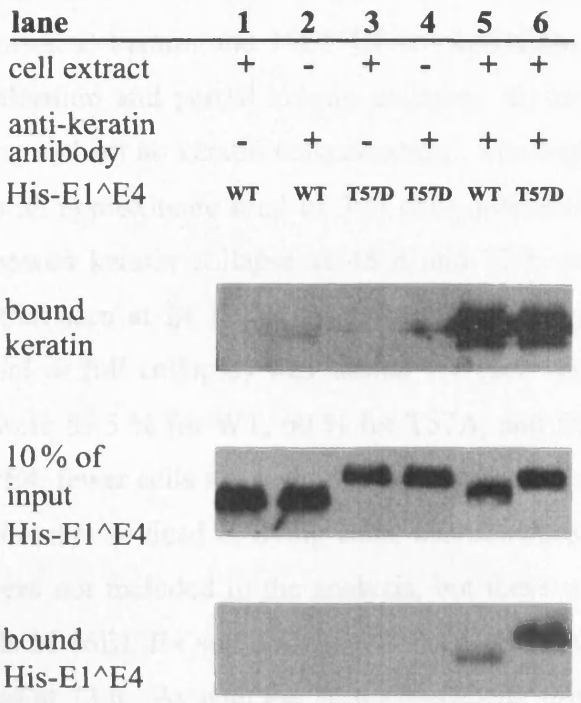


Figure 5.1 Binding of His-E1^E4 to immunoprecipitated keratin
Keratins 8 and 18 were immunoprecipitated from SiHa cells using the L2A1 anti-keratin antibody. A solution of WT or T57D His-E1^E4 was added to the keratin-bound beads (middle panel: Western blot of 10 % of the total His-E1^E4 added). Following a 2 h incubation with His-E1^E4, the beads were washed and the bound keratin and E1^E4 analysed by Western blotting using a rabbit polyclonal anti-keratin antibody (8592) and TVG402 respectively.

SiHa cells were transfected with pMV11 constructs expressing WT, T57A or T57D 16E1^E4. Cells were harvested at 48 h or 72 h post-transfection and immunostained to detect keratin and 16E1^E4. 16E1^E4-positive cells were counted and classed into one of four categories: i) keratin and 16E1^E4 colocalisation and no keratin collapse, ii) keratin colocalisation and partial keratin collapse, iii) keratin colocalisation and full keratin collapse and iv) no keratin colocalisation. The experiments were carried out in triplicate with an approximate total of 300 cells analysed for each type of 16E1^E4. More cells showed keratin collapse at 48 h and 72 h post-transfection (Figure 5.2) compared to that seen at 24 h (Figure 4.7b). The total percentage of cells showing collapse (partial or full collapse) was similar for each type of 16E1^E4 (at 48 h, the mean values were 55.5 % for WT, 60 % for T57A, and 58.25 % for T57D). For each type of 16E1^E4, fewer cells showed collapse at 72 h compared to 48 h. At 72 h, there was a higher number of dead or dying cells, characterised by their fragmented nuclei. These cells were not included in the analysis, but these were the cells that frequently had high levels of 16E1^E4 and collapsed keratin, explaining the apparent decrease in keratin collapse at 72 h. As with the 24 h experiment, this experiment did not include the analysis of very low-level 16E1^E4 that is difficult to visualise by immunostaining.

5.4 T57A shows less colocalisation with cyclin A than WT does

To test whether threonine 57 is important for cyclin A binding, SiHa cells were transfected with pMV11 expressing either WT or T57A 16E1^E4. The cells were harvested and fixed at 48 h post-transfection and then immunostained to detect 16E1^E4 and cyclin A. The 48 h timepoint (post-transfection) was chosen as it was previously found to be optimal for 16E1^E4 and cyclin A colocalisation (Davy et al., 2006). Cells that were positive for both 16E1^E4 and cyclin A were analysed for the presence or absence of cyclin A-16E1^E4 colocalisation. The experiments were performed in triplicate, with an approximate total of 200 cells analysed for each type of 16E1^E4. It was found that an average of 82 % of the WT-containing cells showed some degree of cyclin A colocalisation, while the figure was only 44 % for the T57A mutant (Figure 5.3). This difference was statistically significant ($t_3 = 3.84$, $P = 0.031$).

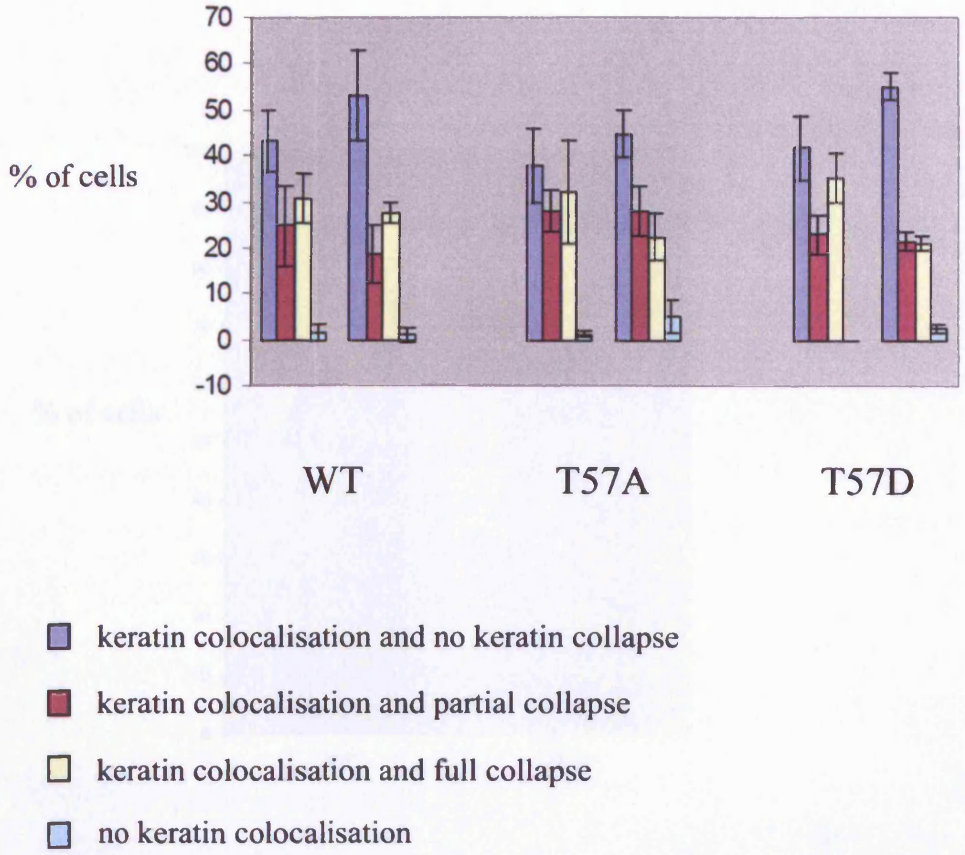


Figure 5.2 Keratin and E1^E4 colocalisation at 48 h and 72 h post-transfection
SiHa cells were immunostained for E1^E4 and keratin at 48 h and 72 h post-transfection of WT, T57A and T57D constructs. The staining patterns were classed into four categories: 'keratin and E1^E4 colocalisation and no keratin collapse', 'keratin colocalisation and partial collapse', 'keratin colocalisation and full collapse' or 'no keratin colocalisation'. The percentage of cells in each category were recorded and the mean values represented in a bar graph. The error bars indicate the standard deviation.

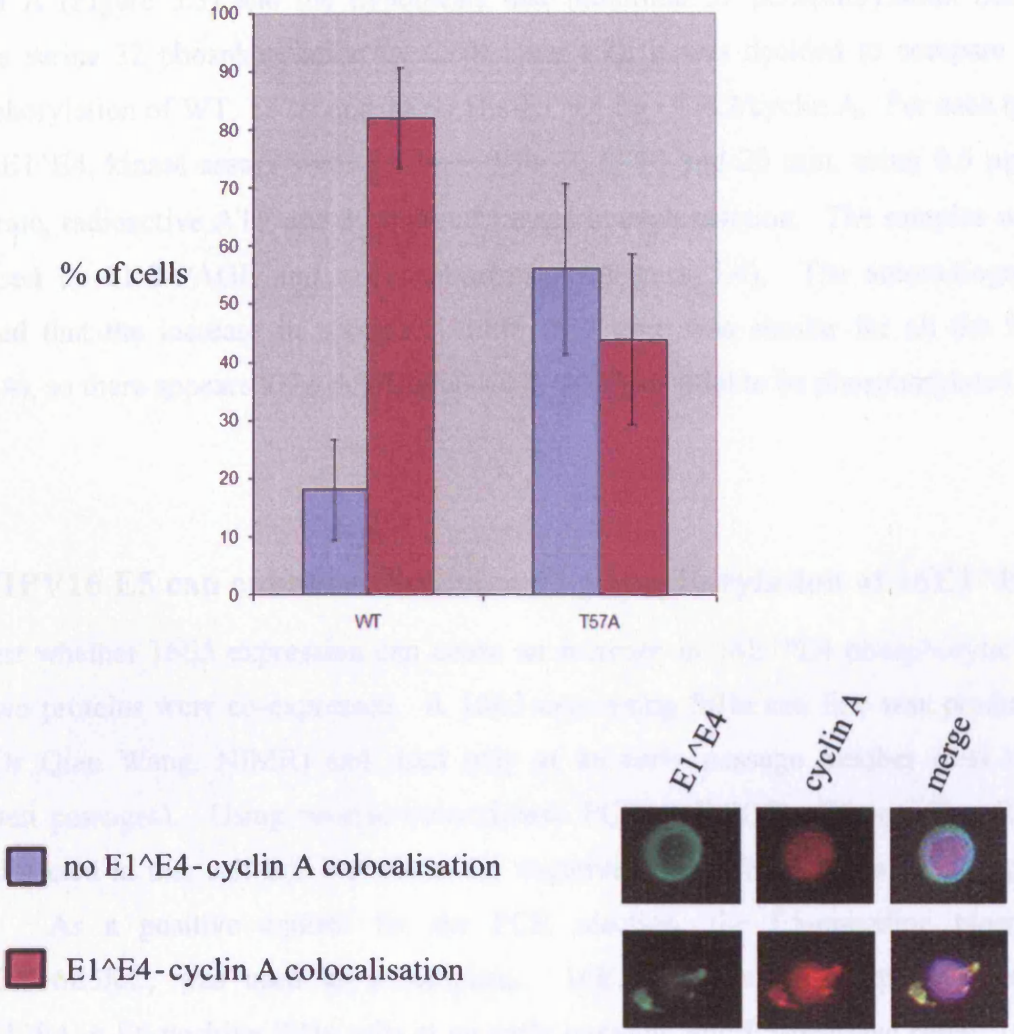


Figure 5.3 Colocalisation of cyclin A with WT and T57A E1^E4
SiHa cells were transfected with WT or T57A constructs and analysed at 48 h post-transfection for E1^E4 and cyclin A colocalisation. An example of non-colocalisation and an example of colocalisation are shown above, with E1^E4 in green and cyclin A in red. The percentage of cells showing colocalisation and non-colocalisation were recorded and the mean values represented in a bar graph. The error bars represent the standard deviation.

5.5 WT, T57A and T57D are phosphorylated by CDK2/Cyclin A at a similar rate

Following the observation that the T57A mutant shows reduced colocalisation with cyclin A (Figure 5.3) and the hypothesis that threonine 57 phosphorylation occurs before serine 32 phosphorylation by CDK (See 4.7), it was decided to compare the phosphorylation of WT, T57A and T57D His-E1^E4 by CDK2/cyclin A. For each type of 16E1^E4, kinase assays were performed for 2, 5, 10 and 20 min, using 0.5 μg of substrate, radioactive ATP and 30 units of kinase in each reaction. The samples were analysed by SDS-PAGE and a PhosphorImager (Figure 5.4). The autoradiograph showed that the increase in phosphorylation over time was similar for all the His-E1^E4s, so there appears to be no differences in their potential to be phosphorylated.

5.6 HPV16 E5 can enhance threonine 57 phosphorylation of 16E1^E4

To test whether 16E5 expression can cause an increase in 16E1^E4 phosphorylation, the two proteins were co-expressed. A 16E5-expressing SiHa cell line was produced (by Dr Qian Wang, NIMR) and used only at an early passage number (less than nine/ten passages). Using reverse-transcriptase PCR (RT-PCR), E5-encoding RNA was detected in this cell line but not in the negative untransfected SiHa cells (Figure 5.5a). As a positive control for the PCR reaction, the E5-encoding plasmid, pMT3H16E5KC, was used as a template. 16E1^E4 was then expressed using rAdE1^E4 in E5-positive SiHa cells at an early passage and E5-negative cells. 16E5 has been shown to activate MAPK in the presence of EGF or sorbitol (Crusius et al., 1997; Crusius et al., 2000), so at 24 h post-infection, the SiHa cells were incubated with 10 ng/ml EGF or 0.5 M sorbitol for 10 min. Then the cells were harvested and prepared for analysis by SDS-PAGE and Western blotting. Samples were run on a 12 % gel for detection of active p42/p44MAPK and GAPDH and on a 15 % gel for separation of the upper and lower bands of 16E1^E4. The anti-ACTIVE® MAPK antibody recognises phosphorylated T183/Y185 of p42MAPK and phosphorylated T202/Y204 of p44MAPK. These residues are phosphorylated by MEK and this leads to MAPK

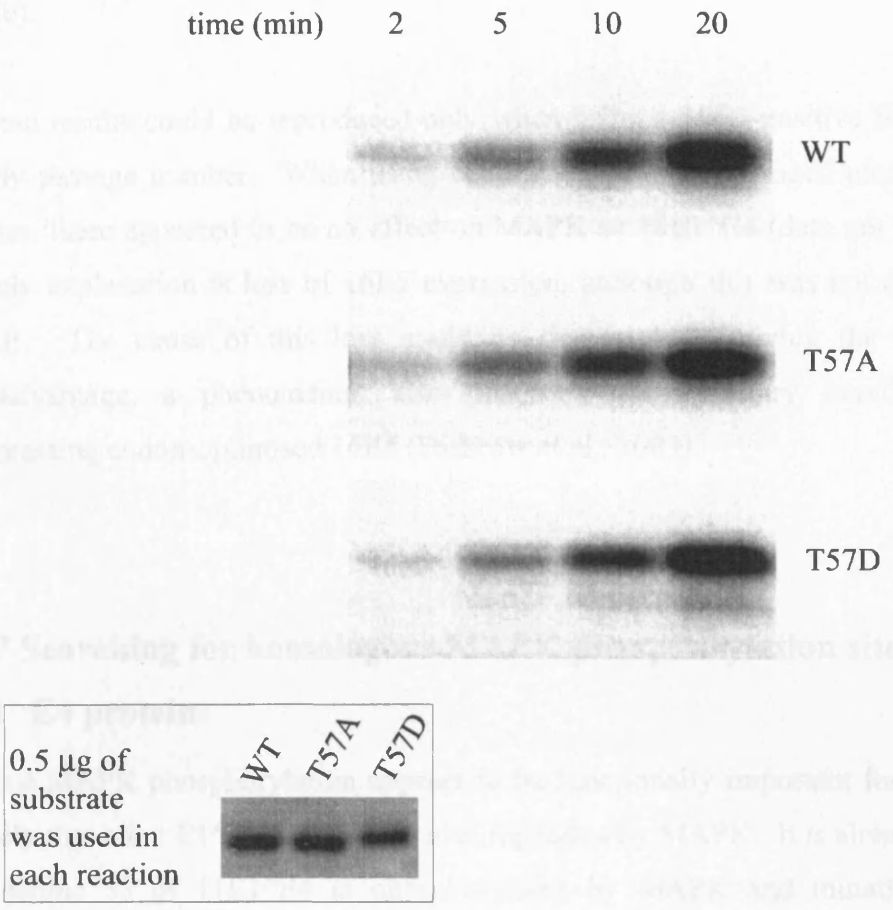


Figure 5.4 *In vitro* phosphorylation of WT, T57A and T57D His-E1^E4 by CDK2/cyclin A

Autoradiograph following CDK2/cyclin A *in vitro* kinase assays using 0.5 µg of WT, T57A or T57D His-E1^E4 as substrates. The inset shows a silver stain of 0.5 µg of each substrate. The reactions were carried out for 2, 5, 10 and 20 min for each type of substrate.

activation. The results showed that the level of active p42/p44MAPK was higher in the E5-positive cells than in the E5-negative cells (Figure 5.5b). As expected, a consequence of this was that T57 phosphorylation of 16E1^E4 was increased, as shown by an increased intensity of the upper bands in relation to the lower bands (Figure 5.5b).

These results could be reproduced only when using a 16E5-positive SiHa clone at an early passage number. When using cells that had been passaged more than nine/ten times, there appeared to be no effect on MAPK or 16E1^E4 (data not shown) and the likely explanation is loss of 16E5 expression, although this was not checked by RT-PCR. The cause of this loss could be due to 16E5 giving the cells a growth disadvantage, a phenomenon also observed with primary keratinocytes stably expressing codon-optimised 16E5 (Disbrow et al., 2003).

5.7 Searching for homologous MAPK phosphorylation sites in other E1^E4 proteins

Since MAPK phosphorylation appears to be functionally important for 16E1^E4, it is likely that other E1^E4 proteins are also regulated by MAPK. It is already known that threonine 53 of 11E1^E4 is phosphorylated by MAPK and mutating this residue reduces the filamentous appearance of the protein and inhibits multimerisation (Bryan et al., 2000). E1^E4 sequence alignments were therefore performed to check for the presence of MAPK sites (P-X-S/T-P, X-X-S/T-P) homologous to threonine 57 of 16E1^E4 or threonine 53 of 11E1^E4.

Interestingly, for HPV16, it appears that E5 can increase E1^E4 phosphorylation through the activation of the MAPK pathway, so it is possible that the E5 protein of other HPV types have also evolved to increase the phosphorylation of E1^E4. Recently, Schiffman et al. (2005) conducted a phylogenetic analysis of anogenital/mucosal HPVs and noted whether or not each HPV type contained an E5 ORF (see Appendix.6). To see whether there is a relationship between E5 presence and an E1^E4 MAPK site, alignments were performed, firstly using 16E1^E4 and 11E1^E4 and five other E1^E4 types from other E5-positive viruses, and then using 16E1^E4

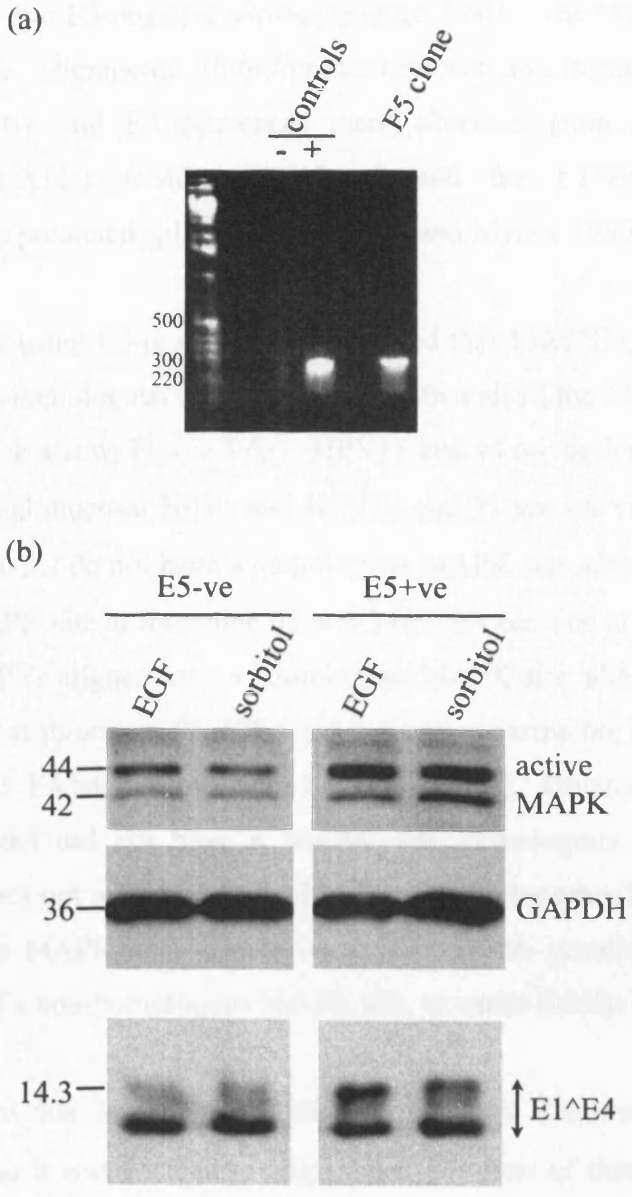


Figure 5.5 Effects of E5 on MAPK and E1^E4 phosphorylation
(a) Agarose gel electrophoresis showing RT-PCR products; E5-coding mRNA was detected from a SiHa clone stably transfected with an E5-expressing vector. The negative control used untransfected SiHa cells and the positive control used the E5-encoding plasmid, pMT3H16E5KC, as PCR templates. (b) Western blots of activated p42 and p44 MAPK, GAPDH loading controls and E1^E4, following treatment of rAdE1^E4-infected E5-positive and E5-negative SiHa cells with EGF or sorbitol.

and 11E1^E4 and five E5-negative viruses (Figure 5.6a). The 'Multalin' programme was used for the alignments (http://prodes.toulouse.inra.fr/multalin/multalin.html; Corpet, 1988). E1 and E4 sequences were obtained from the HPV database (http://www. stdgen.lanl.gov/stdgen/virus/hpv/) and the E1^E4 sequences were generated using the predicted splice sites (Doorbar and Myers, 1996).

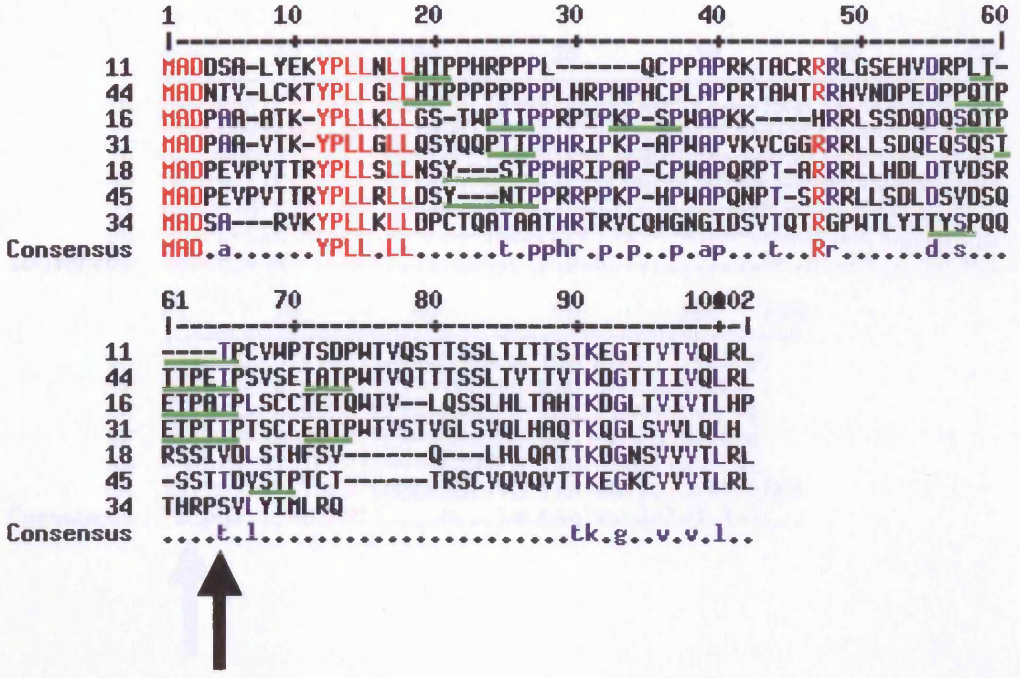
The first alignment using E5-positive types revealed that 11E1^E4, 44E1^E4 16E1^E4 and 31E1^E4 have homologous threonine residues that all fit the MAPK consensus site (indicated by a black arrow; Figure 5.6a). HPV11 and 44 are both members of the α10 species of anogenital/mucosal HPVs and HPV16 and 31 are α9 viruses. HPV18, 45 (α7) and HPV34 (α11) do not have a homologous MAPK site, although 45E1^E4 does have a nearby MAPK site at threonine 63 and 34E1^E4 has one at serine 54. None of the E5-negative HPVs aligned have a homologous MAPK site, although 54E1^E4 does have a nearby site at threonine 62, 57E1^E4 has one at serine 66, 61E1^E4 has one at serine 63 and 29E1^E4 has one at serine 70 (Figure 5.6a). Given that three of the E5-positive types tested did not have a MAPK site homologous to threonine 57 of 16E1^E4, there does not appear to be a clear correlation between E5 and the presence of the homologous MAPK site. However, it is of course possible that E5 enhances phosphorylation of a non-homologous MAPK site, to cause similar biological effects.

The E1^E4 proteins that do possess a homologous MAPK site, are members of the α9 and α10 species, so it was decided to align other members of these species with each other (Figure 5.6b). The first alignment is of α9 members and shows that 31, 35, 52 and 58E1^E4 have a MAPK site homologous to threonine 57 of 16E1^E4. 33E1^E4 does not have a homologous residue but has nearby MAPK consensus sites at serine 43 and threonine 52. The second alignment is of α10 members and shows that 6, 13, 44 and 55E1^E4 have a MAPK site homologous to threonine 53 of 11E1^E4. Interestingly, fifteen of the nineteen E1^E4 sequences analysed possess a threonine residue that aligns with threonine 57 of 16E1^E4, although only ten of them (including 16E1^E4) are in MAPK consensus sites. None of the proteins with a homologous MAPK site has a serine residue at that site, despite the fact that MAPK can phosphorylate both serines and threonines. This suggests that there is functional importance of the unphosphorylated threonine as well as roles for phosphorylated

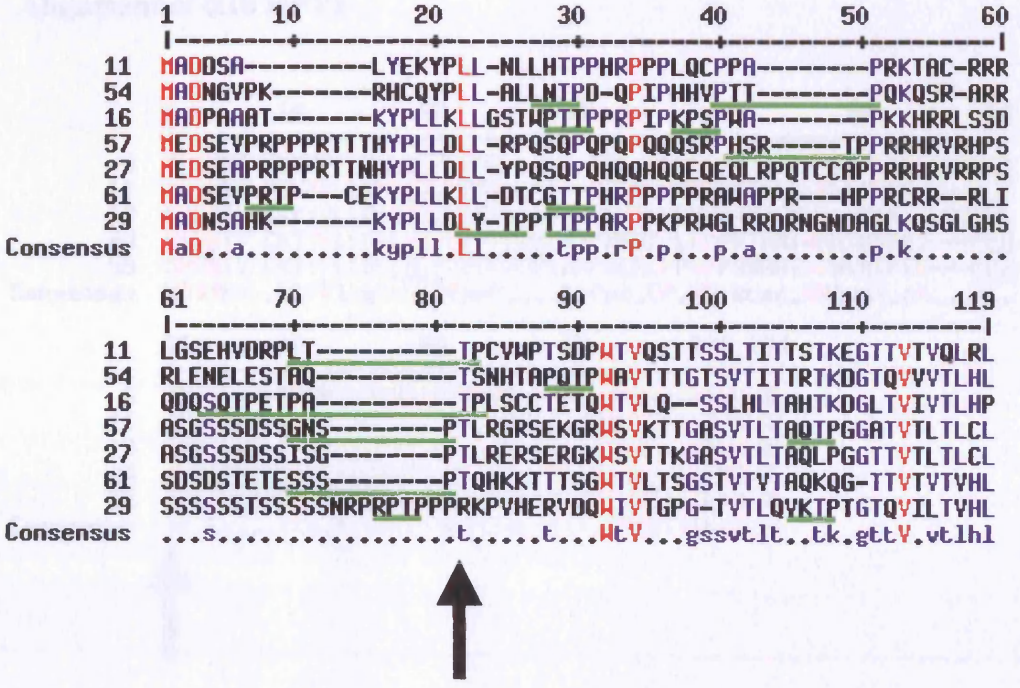
Figure 5.6 E1^E4 amino acid sequence alignments
E1^E4 sequences were aligned using MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html) to search for MAPK phosphorylation sites homologous to HPV11 and HPV16. The region homologous to the MAPK site of 11E1^E4 and 16E1^E4 is indicated by an arrow. All MAPK consensus sites are underlined in green. (a) In the top alignment, HPV11 and HPV16 were aligned with examples of anogenital HPVs (α species) that were described as having an E5 ORF by Schiffman et al. (2005). HPVs 11 and 44 are α10 viruses, HPVs 16 and 31 are α9 virus, HPV34 is an α11 virus, and HPVs 18 and 45 are α7 viruses. In the bottom alignment, HPVs 11 and 16 were aligned with examples of types that do not have a homologous E5 ORF. HPV54 is an α13 virus, HPVs 57 and 27 are α4 viruses, HPV61 is an α3 virus and HPV29 is an α2 virus. (b) In the top alignment, 16E1^E4 was aligned with other α9 E1^E4s, and in the bottom alignment, 11E1^E4 was aligned with other α10 E1^E4s.

(a)

HPV11 and HPV16 aligned with E5-positive HPVs

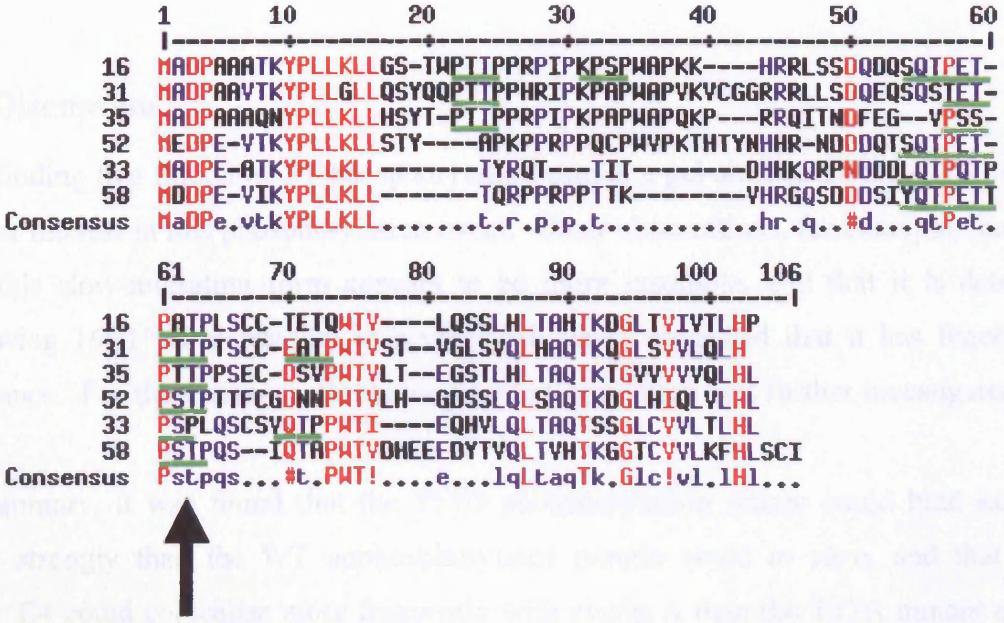


HPV11 and HPV16 aligned with E5-negative HPVs

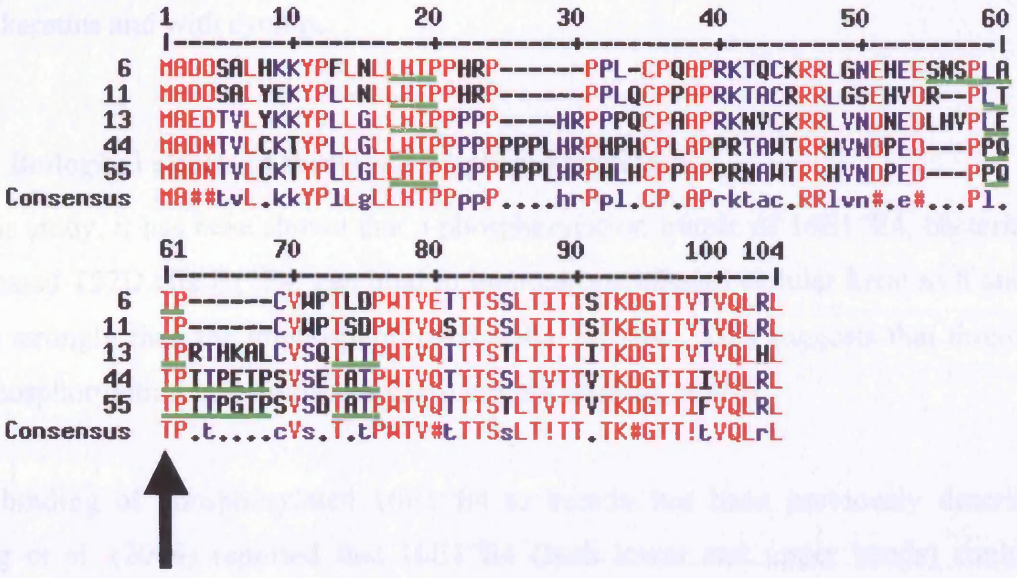


(b)

Alignment of α9 HPVs



Alignment of α10 HPVs



threonine and a serine residue may not substitute. Alternatively, a serine residue may not be tolerated because of its effect on the E2 ORF (which overlaps that of E4).

5.8 Discussion

The finding that threonine 57 phosphorylation causes a gel-shift in SDS-PAGE, led to further interest in this phosphorylation event. Other observations, for example, the fact that this slow-migrating form appears to be more insoluble, and that it is detected following 16E1^E4 expression in several cell lines, suggested that it has functional relevance. For these reasons, threonine 57 phosphorylation was further investigated.

In summary, it was found that the T57D phosphorylation mimic could bind keratin more strongly than the WT unphosphorylated protein could *in vitro,* and that WT 16E1^E4 could colocalise more frequently with cyclin A than the T57A mutant could in SiHa cells. In addition, 16E5 has the ability to increase threonine 57 phosphorylation of 16E1^E4 in SiHa cells through activation of p42/p44MAPK. Taken together, these results suggest that threonine 57 phosphorylation may be regulated *in vivo* and has important functional effects. It can be hypothesised that 16E5 activates 16E1^E4 phosphorylation *in vivo* which leads to an increase in 16E1^E4 association with keratins and with cyclins.

5.8.1 Biological effects of threonine 57 phosphorylation

In this study, it has been shown that a phosphorylation mimic of 16E1^E4, bacterially-expressed T57D His-E1^E4, can bind to immunoprecipitated cellular keratins 8 and 18 more strongly than the unphosphorylated 16E1^E4 can. This suggests that threonine 57 phosphorylation may cause increased keratin binding *in vivo.*

The binding of phosphorylated 16E1^E4 to keratin has been previously described. Wang et al. (2004) reported that 16E1^E4 (both lower and upper bands) could be coimmunoprecipitated from the NP40-soluble fraction of 16E1^E4-expressing cells using an anti-keratin 8/18 rabbit serum. When the cells were not treated with OA (a phosphatase inhibitor), only the lower band (13 kDa) of 16E1^E4 was detected. In this study, it has been found that the threonine 57-phosphorylated upper band (14 kDa) is

predominantly found in the insoluble fraction (see 4.6.2), so in the experiment by Wang at al. (2004), the upper band may have detected because only the NP40-soluble fraction was analysed. However, when the cells were treated with OA, the upper band was present in the NP40-soluble fraction, as only the upper band was coimmunoprecipitated. The explanation could be that the cells contained more of the upper band form than the lower band form following OA treatment. Alternatively, the upper band had bound to keratin with greater affinity than the lower band did. The latter would support the findings in this study.

To further investigate the possibility that threonine 57 phosphorylation can regulate keratin binding, cultured cells were transfected with WT, T57A and T57D in order to visualise keratin-16E1^E4 patterns by immunostaining. However, the immunostaining patterns did not reveal a stronger association of T57D with keratin compared to WT or T57A with keratin. It could be that this phenomenon is specific to histidine-tagged T57D as the histidine tag was present in the *in vitro* experiment but not in the transfection experiment, or it may be that cellular factors dampen the ability of T57D to form strong associations with keratin. The more likely explanation is that in the transfection experiments, the vast majority of the 16E1^E4 was already bound to keratin, so that any increased ability of T57D to bind keratin was not detectable. The level of 16E1^E4 was perhaps too high to be able to detect subtle differences in activity between the WT, phosphorylation mutant and phosphorylation mimic, since expression was from a CMV promoter. The alternative could be to express 16E1^E4 from the HPV16 genome or a system where transcription can be better controlled, such as the tet-on system where transcription can be activated by doxycyclin in a dose-dependent manner (Gossen et al., 1995).

Interestingly, the fact that biological effects of phosphorylation can be affected by the method of protein expression, has been previously described for BPV1 E2 (McBride and Howley, 1991). Serine 301 of BPV1 E2 is phosphorylated by CKII and functional effects of substituting serine 301 with alanine (S301A) were investigated. When E2 was expressed in CV-1 cells (north African green monkey fibroblast cell line) from a plasmid containing a SV40 promoter, there were no differences in transactivation ability between WT and S301A E2. However, when BPV1 genomes were used in the same transactivation assays, the genome containing the S301A mutation caused greater

transactivation than the WT genome did. S301A BPV1 E2 shows increased transactivation (and viral DNA replication) because it is targeted less by the ubiquitin-proteasome pathway in comparison to WT, so possesses a longer half-life than WT does. However, expression of E2 from an SV40 promoter had led to protein levels that were too high to detect effects of the change in protein turnover.

The observation that cyclin A colocalisation is lower for the T57A mutant compared with the WT protein, is another phenomenon that may become more apparent when lower levels of 16E1^E4 are analysed. 16E1^E4 has been shown to cause cell-cycle arrest in G_2 (Davy et al., 2002) and the mechanism is likely to be through binding of cyclins (Davy et al., 2006; Davy et al., 2005). The possible role of threonine 57 itself or threonine 57 phosphorylation in cell-cycle arrest is therefore important to investigate. Ultimately, it will be necessary to analyse the phosphorylation mutant and the phosphorylation mimic in the context of the viral genome, ideally in a raft system which represents an infected epithelium.

It was already known that CDK2/cyclin A can phosphorylate WT 16E1^E4 at serine 32 *in vitro* (see Chaper 3). Now it was important to answer whether the T57A mutant has a reduced affinity for this kinase complex and would therefore be phosphorylated at a slower rate. This would give an explanation for the reduced cyclin A binding. Also, since data in Chapter 4 (see 4.7) suggested that threonine 57 phosphorylation may occur before serine 32 phosphorylation, it was considered important to test whether the T57D phosphorylation mimic would be phosphorylated at serine 32 at a faster rate compared to WT. CDK2/cyclin A *in vitro* kinase assays were therefore performed using WT, T57A and T57D His-E1^E4 as substrates. The results showed, however, that at least *in vitro*, T57A does not have a reduced ability to be phosphorylated by CDK2/cyclin A compared to WT, and T57D is not phosphorylated more easily.

The finding that T57A colocalises with cyclin A less frequently than WT, may therefore be independent of its ability to be phosphorylated by CDK2/cyclin A. The T57A mutant may be phosphorylated just as readily but have a lowered cyclin A binding potential. This could be confirmed by *in vitro* binding experiments for example by using GST-tagged WT and T57A 16E1^E4 to pull-down cyclin A from cells. Similar experiments were performed to show binding between cyclin B and

16E1^E4 (Davy et al., 2005). Alternatively, the reason for the apparent reduction in T57A binding to cyclin A could be a secondary effect of the activity of the T57A mutant. For example, if T57A differs from WT in the way it forms multimers or associates with keratins or other cellular proteins, then the degree of cyclin colocalisation may be affected.

In a previous screen for the ability of 16E1^E4 mutants to induce G_2-arrest in *S. pombe*, threonine 57 was not shown to be required for arrest since a Δ52-57 mutant could still cause arrest (Davy et al., 2002). Threonine 57 itself or threonine 57 phosphorylation may not be essential for cyclin binding and G_2-arrest, but it may act as an stimulatory factor for these events. The residue that was found to be crucial for cyclin binding and G_2-arrest was threonine 23 (Davy et al., 2005; Davy et al., 2002).

The fact CDK2/cyclin A phosphorylates the T57D mutant and the WT protein equally well, suggests that threonine 57 phosphorylation is not required for serine 32 to be phosphorylated by CDK2/cyclin A. The absence of detectable serine 32 phosphorylation in the mono-phosphorylated 16E1^E4 extracted from SiHa cells (see 4.7) may therefore be due to 16E1^E4 having a greater affinity for MAPK than for CDK/cyclin or because MAPK activity levels are high in these cells. Alternatively, the T57D mutant may not be resembling phosphorylation appropriately so may not be behaving in the same way as threonine 57-phosphorylated 16E1^E4 (see 5.8.2).

5.8.2 Does the T57D mutant mimic threonine 57 phosphorylation?

The T57D mutant migrates slower than WT 16E1^E4 in a similar fashion to threonine 57-phosphorylated 16E1^E4 (Figure 4.6a). This suggests that this mutant protein has a similar structural conformation to the threonine 57-phosphorylated form. It is therefore reasonable to assume that any behaviour of threonine 57-phosphorylated 16E1^E4 that is dependent on the tertiary structure of the protein, can be reproduced with the T57D mutant. Any activity dependent on the negatively charged phosphate group should also be reproducible by substitution with aspartic acid. However, an activity sensitive to the exact size of phosphorylated threonine 57 or dependent on threonine atoms as well as the phosphate group, may not be mimicked by aspartic acid. In this case, glutamic acid may be a better substitute or it may not be possible to mimic phosphorylation.

It is important to consider that glutamic acid may mimic phosphorylated threonine 57 better than aspartic acid can. Indeed, there are several reports of differing biological effects depending on whether aspartic acid or glutamic acid have been used to mimic phosphorylation in proteins (Carlson et al., 2001; Gradin et al., 2002). Therefore, for future projects, the activity of phosphorylated 16E1^E4 should be compared to both aspartic acid and glutamic acid mutants.

5.8.3 Regulation of 16E1^E4 phosphorylation by 16E5

The level of threonine 57 phosphorylation was higher when 16E1^E4 was expressed in a 16E5-expressing cell line compared with a 16E5-negative cell line. This was co-incident with the activation of p42/44MAPK. The increase in 16E1^E4 phosphorylation is therefore a novel role for 16E5. The fact that an increased level of endogenous active p42/44MAPK was associated with increased 16E1^E4 phosphorylation, provides further support that these kinases phosphorylate 16E1^E4.

E5 proteins may therefore be important for E1^E4 activity *in vivo*. Indeed, a 31E5 knockout genome appeared to have an effect on 31E1^E4 levels (Fehrmann et al., 2003). Rafts supporting the HPV31 life cycle showed a reduced number of 31E1^E4-positive cells when 31E5 was mutated, as detected by immunostaining. Also, after methylcellulose treatment of cells, 24 % of WT-containing cells stained for 31E1^E4, compared with 14.5 % for mutant 31E5-containing cells. It would be interesting to analyse the extent of 31E1^E4 phosphorylation in the presence or absence of 31E5 to decipher whether 31E1^E4 phosphorylation is related to its levels. 31E1^E4 does in fact have a MAPK consensus site homologous to threonine 57 of 16E1^E4 (Figure 5.6).

It could be that effects of E5 on E1^E4 may be different in different HPV types, since loss of 16E5 did not cause a decrease in 16E1^E4 levels in HPV16-containing rafts (Genther et al., 2003). Whether 16E5 co-expression or mutation affects 16E1^E4 activities such as keratin or cyclin association or cell cycle arrest could be investigated in future experiments.

It was interesting to consider that E5 roles may be conserved between different HPVs and there is a requirement for E5 activity in order to achieve E1^E4 phosphorylation by MAPK. Examples of E5-positive and E5-negative HPVs were analysed to see if more E5-positive HPVs had a MAPK site homologous to threonine 57 of 16E1^E4. This was the case, but not all of the E5-positive types analysed had a homologous MAPK site, so the correlation was weak. However, most E1^E4s analysed, had MAPK consensus sites in neighbouring regions so it is likely that E5 can still activate phosphorylation by MAPK to cause a structural change, but not necessarily at a site homologous to threonine 57 of 16E1^E4.

5.8.4 Homologous phosphorylation sites

Of the E1^E4 types analysed, those found to possess MAPK sites homologous to threonine 57 of 16E1^E4, are the α9 types, 31, 35, 52 and 58 and the α10 types, 6, 11 (already shown to be a MAPK site *in vivo* by Bryan et al. (2000)), 13, 44 and 55. The α9 viruses are all high- risk types that can cause cancer whereas the α10 viruses are rarely associated with malignancy. However, both types of viruses may regulate their E1^E4 activites through MAPK phosphorylation. HPVs 11, 31 and 45 have been analysed for E1^E4-keratin association, and colocalisation has been detected in cell culture (personal communication, Peter Laskey). Bryan et al. (2000) described how mutating the MAPK site in 11E1^E4 caused the protein to have a diffuse distribution instead of a filamentous pattern. Therefore, E5-enhanced, MAPK phosphorylation may affect keratin binding of other E1^E4s in the same way that it is thought to affect keratin binding of 16E1^E4.

Interestingly, the other phosphorylation sites mapped in this study (CDK site at serine 32, PKA site at serine 43) are not conserved between 16E1^E4 and other α9 members. Of course, CDK and PKA could phosphorylate other E1^E4s in non-homologous sites. The threonine 57 MAPK site perhaps is more frequently conserved between different E1^E4s, compared to other phosphorylation sites, because the biological effects of this phosphorylation event are dependent on a structural change. For a conformational change to occur, surrounding amino acids are involved, so the location of the phosphorylation site is important. MAPK phosphorylation at a non-homologous site

may not produce a similar structural change and therefore not have the same functional effects.

Chapter 6: Final Discussion

The objectives of this study were to investigate whether 16E1^E4 is phosphorylated in cells, to map the phosphorylation site(s) and to decipher the role of 16E1^E4 phosphorylation in the virus life cycle. The outcomes of the study have included the characterisation of several phosphorylation sites of 16E1^E4 and the finding that the protein is phosphorylated by at least two kinases in cells; CDK1/2 and p42/p44MAPK. Phosphorylation by p42/p44MAPK induces a conformational change in 16E1^E4 (personal communication, Dr Pauline McIntosh, NIMR) and is important for regulation of keratin binding and cyclin colocalisation. Furthermore, the level of 16E1^E4 phosphorylation can be enhanced by activation of p42/p44MAPK by 16E5.

By improving our understanding of the regulation of HPV proteins by phosphorylation, there will be increased opportunity to develop agents that interefere with this regulation. As a consequence, this may lead to the development of novel therapies to treat HPV infections and HPV-related cancers.

6.1 Kinases involved in E1^E4 phosphorylation

The HPV life cycle is dependent on the differentiation status of keratinocytes in the epithelium, and protein kinase signalling is integral to the growth, differentiation and survival of keratinocytes. As well as regulating keratinocyte differentiation to ensure suitable conditions for viral growth, protein kinases can influence HPV more directly by phosphorylation of viral proteins.

Previously, 1E1^E4 has been shown to be phosphorylated by PKA (Grand et al., 1989), and 11E1^E4 has been found to be phosphorylated by both PKA and p42MAPK (Bryan et al., 2000). This study has shown that the E1^E4 protein of HPV16 can be phosphorylated *in vitro* by CDK1 and CDK2 at serine 32, by p42MAPK at threonine 57, by PKA at serine 43, and by PKCα at a site between amino acids 35 and 59. Of these events, phosphorylation of threonine 57 and serine 32 occurs when the protein is expressed in a HPV16-transformed cervical cancer cell line (SiHa cells), and p42/44MAPK and CDK1/2 are the respective kinases involved.

It is important to consider that *in vivo*, the 16E1^E4 phosphorylation status may vary depending on the differentiation status of the epithelium and on which other HPV proteins are active. All the kinases discussed above are expressed in the epithelium and are important in keratinocyte signal transduction. These kinases and their activities in normal and HPV-infected keratinocytes are reviewed below.

6.1.1 Kinases in normal epithelium

PKC

Of the nine PKC genes in humans, five (α, δ, ε, η, ζ) are expressed in keratinocytes (Reynolds et al., 1994). PKCα is a classical PKC isoform, meaning that it is activated by calcium and by diacylglycerol or phorbol esters. PKCδ, ε and η are novel isoforms (calcium-independent but activated by diacylglycerol/phorbol esters), while PKCζ is an atypical isoform (independent of both calcium and diacylglycerol/phorbol esters). PKCα is found in the suprabasal layer where it triggers irreversible growth arrest (Bollag et al., 1993; Tibudan et al., 2002). Furthermore, PKCα activity is required for expression of differentiation markers such as involucrin (Yang et al., 2003). The activation of other PKC isoforms, in particular, PKCη (Kashiwagi et al., 2002) and PKCδ (Deucher et al., 2002), have also been shown to be necessary for expression of keratinocyte terminal differentiation markers.

The importance of PKC in epithelial differentiation is demonstrated by the fact that PKC activators are necessary for ensuring a complete keratinocyte differentiation programme in raft cultures (Meyers et al., 1992). It is also required for capsid protein expression (Meyers et al., 1992; Pray and Laimins, 1995) and the treatment of HPV31-containing rafts with a synthetic diacylglycerol, even caused a slight increase in E1^E4 levels (Pray and Laimins, 1995).

CDK

CDKs and their associated cyclins are active in all proliferating cells, so are important in the basal layer of the epithelium. In order to exit from the cell cycle and progress towards differentiation, CDKs and cyclins must be regulated. In a study where keratinocytes in culture were induced to differentiate using high extracellular calcium, D-type cyclins, cyclin E and CDK2 protein levels decreased (along with associated

kinase activity), but CDK4 and CDK6 levels remained unchanged (Martinez et al., 1999). In the same study, the interactions between CDK inhibitors and CDKs was also investigated. It was found that upon differentiation, all three CDK inhibitors analysed (p21, p27 and p57), increased the extent to which they associated with CDK4, CDK6 and CDK2. This suggests that downregulation of most CDK/cyclin activity is required for differentiation. In fact, one of the activites of PKCη in keratinocytes is the association with cyclin E/CDK2/p21 complexes and this appears to inactivate CDK2 (Kashiwagi et al., 2000).

MAPK

In keratinocytes, MAPK pathways can be activated by various signals (Figures 1.4 and 4.12a). For example, EGF can activate p42/p44MAPK (Mitev et al., 1995), UVB can stimulate JNK (Assefa et al., 1997) and osmotic stress can activate p38MAPK (Garmyn et al., 2001). All these MAPK isoforms function to regulate keratinocyte proliferation, survival and differentiation. Seo et al. (2004) reported that all these MAPK pathways are induced during calcium-dependent differentiation of keratinocytes, as measured by phosphorylated (active) p42/p44MAPK, p38MAPK and JNK. Inhibition of MEK1/2 (which is upstream of p42/p44MAPK) or p38MAPK prevented expression of differentiation markers such as loricrin and filaggrin, but inhibition of JNK1/2 had no effect. Interestingly, overexpression of PKCα (in low calcium) led to activation of p42/p44MAPK and JNK1/2 but not p38MAPK, as well as expression of differentiation markers. This suggests that *in vivo*, much of the PKCα-induced keratinocyte differentiation could be mediated by MAPK signalling. Similarly, PKCδ and η have been shown to activate p38MAPK and PKCδ activation of involucrin was blocked by expression of a dominant-negative p38MAPK (Efimova et al., 2002).

PKA

PKA has an ubiquitous distribution and is central to many cellular processes such as transcription regulation, ion conductance, membrane trafficking and cell motility (Shabb, 2001). However, the physiological role of PKA in the epithelium is not well understood. PKA is activated by the binding of c-AMP, a secondary messenger generated by G-protein activity. In HaCaT cells, raising the level of intracellular c-AMP led to the expression of the differentiation markers, involucrin, keratins 1 and 10, and transglutaminase (Mammone et al., 1998). Also in the same study, the use of PKA

inhibitors prevented the expression of keratins 1 and 10. PKA can regulate other signal transduction pathways and this is likely to contribute to its effects in the epithelium. For example, it can regulate intracellular calcium by phosphorylation of calcium channels such as inositol 1,4,5 trisphosphate (IP_3) receptors (Nakade et al., 1994; Tertyshnikova and Fein, 1998), and it can inhibit p42/p44MAPK signalling by phosphorylating raf (Dhillon et al., 2002).

6.1.2 Kinase activity in HPV-infected cells

The kinase activity in HPV-infected epithelium is influenced by the activities of the HPV proteins themselves. As previously discussed (see 1.13), several of the viral proteins have effects on the cell cycle or signalling pathways, so they could also affect 16E1^E4 phosphorylation.

Active p42/p44MAPK can be upregulated by 16E5 as observed in this study and previously reported by others (Crusius et al., 1997; Gu and Matlashewski, 1995). E6 of HPVs 16, 18 and 31, can also increase the level of phosphorylated p42/p44MAPK (Chakrabarti et al., 2004). Also, when cDNA microarrays were used to analyse the effects of 16E6 and 16E7 on gene expression in keratinocytes, upregulation of p38MAPK, p42/p44MAPK, CDK1, cyclin B1 and cyclin A was observed (Nees et al., 2001). The activation of CDK2/cyclin A and CDK2/cyclin E by 16E7 has also been reported (previously discussed; see 1.13.2.2).

6.2 The roles of E1^E4 phosphorylation

The E1^E4 protein has been implicated in a variety of roles including cell cycle arrest, viral DNA replication, expression of capsid proteins, and association with and disruption of keratins and cornified envelopes. Cell cycle arrest mediated by cyclin binding, could be necessary to sustain a cellular environment that allows for viral DNA replication, while the disruption of keratins and cornified envelopes could aid virus release from cells. Overall, these functions support completion of the viral life cycle rather than malignancy. Indeed, E1^E4 is absent in high grade cervical biopsies (Middleton, 2003).

The regulation of E1^E4 expression and activities is not well understood. Traditionally, E1^E4 is described as a 'late protein', as it becomes abundant following activation of the differentiation-dependent promoter, and its staining in lesions coincides with the start of L1 expression. Upon closer examination however, it has been found that 16E1^E4 is functionally important for early events such as viral DNA replication. This event occurs in the lower, undifferentiated epithelial layers. In such cells, Western blotting, but not immunostaining, can detect 16E1^E4 (Nakahara et al., 2005). It is therefore important to consider that phosphorylation may have different roles depending on whether 16E1^E4 is at the low level phase or the abundant phase. Also, phosphorylation itself may be a regulator of the change in 16E1^E4 levels/distribution.

This study has found that threonine 57 phosphorylation by p42/p44MAPK induces a structural change in 16E1^E4 (personal communication, Dr Pauline McIntosh, NIMR) that is manifested as a gel-shift in SDS-PAGE. The result is the accumulation of the phosphorylated form in the insoluble fraction of the cell. It is likely that this form has a greater affinity for keratin and accelerates the formation of 16E1^E4 multimers on keratin filaments (Figure 6.1). This hypothesis is based on the ability of the T57D phosphorylation mimic to bind keratin *in vitro* more strongly than the unphosphorylated protein does. p42MAPK phosphorylation of 11E1^E4 is likely to regulate keratin binding in a similar fashion, since mutation of the MAPK site reduces the formation of 11E1^E4-containing filamentous structures (Bryan et al., 2000). Once 16E1^E4 has formed keratin-associated filaments/aggregates, it becomes easier to visualise by immunostaining with TVG405 and appears to be abundant in the cell. *In vivo,* MAPK phosphorylation may therefore act as a switch to trigger the accumulation of 16E1^E4 on keratins. An important future experiment would be to characterise active MAPK expression in the epithelium to see if there is correlation with the onset of high-level 16E1^E4.

16E1^E4 accumulation on keratin is likely to be important for its biological functions. The association with keratin filaments disrupts normal keratin dynamics and *in vivo*, this is manifested by the collapse or reorganisation of keratin networks (Doorbar et al., 1991; Wang et al., 2004). This is hypothesised to weaken the cell and aid virus release. The association of 16E1^E4 with keratins may also facilitate other 16E1^E4 activities,

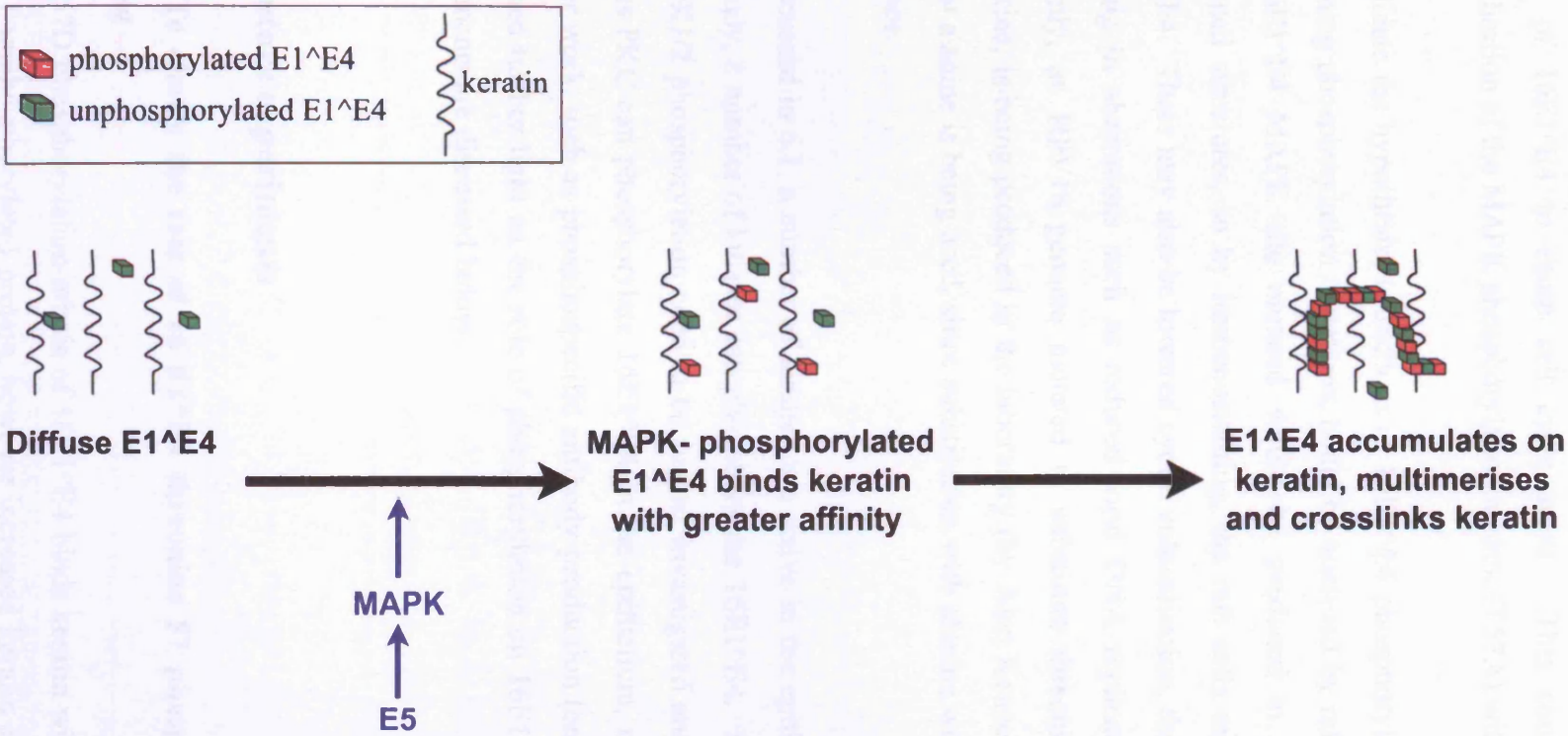


Figure 6.1 A model for the accumulation of E1^E4 on keratin

The hypothesis for the effect of p42/p44MAPK phosphorylation of E1^E4 is illustrated. Following E5-enhanced phosphorylation of E1^E4 by MAPK, there is preferential binding of the phosphorylated E1^E4 to keratin. This allows E1^E4 to accumulate on keratins, causing the disruption of keratin dynamics as described by Wang et al. (2004). The accumulation of E1^E4 on keratin may facilitate other E1^E4 functions

given that keratins are increasingly regarded as a 'scaffold' for many cellular events. For example, keratin binding or the way phosphorylated 16E1^E4 arranges itself on keratins, may allow cyclins A or B to be tethered more tightly, thus increasing the ability of 16E1^E4 to cause cell cycle arrest. This could explain the poorer colocalisation of the MAPK phosphorylation mutant (T57A) with cyclin A.

To validate the hypothesised functions of 16E1^E4 phosphorylation, HPV16 genomes containing phosphorylation mutations, could be analysed by raft culture. A virus with the 16E1^E4 MAPK site mutated would be predicted to have reduced keratin-associated structures, so by immunostaining, the raft cells may appear to have less 16E1^E4. There may also be lowered cyclin colocalisation, thus less cell cycle arrest, resulting in aberrations such as reduced viral DNA replication and amplification. Currently, an HPV16 genome mutated to substitute threonine 57 of E1^E4 with isoleucine, is being produced in the laboratory (by Alan Kennedy, NIMR). Isoleucine and not alanine is being used, since substitution with alanine would alter the E2 protein sequence.

As discussed in 6.1, a number of kinases are active in the epithelium, and as found in this study, a number of kinases can phosphorylate 16E1^E4. The biological functions of CDK1/2 phosphorylation need to be further investigated and whether other kinases such as PKC can phosphorylate 16E1^E4 in the epithelium, needs to be determined. Further work, such as phosphospecific antibody production (ongoing, see Appendix.5) will shed further light on the role of phosphorylation on 16E1^E4 regulation. Future experiments are discussed below.

6.3 Future experiments

6.3.1 To clarify the role of 16 E1^E4 threonine 57 phosphorylation in keratin binding

The T57D phosphorylation mimic of 16E1^E4 binds keratin with a higher affinity than the WT unphosphorylated protein, however increased keratin association was not seen when T57D was analysed in SiHa cells. By immunofluorescence, the 16E1^E4 that is easily visible, is the form that has formed structures, such as filamentous structures or

collapsed aggregates. In the immunofluorescence analysis in this study, only cells with visible 16E1^E4 were counted, so perhaps only the cells with keratin-bound 16E1^E4 were included. Therefore, any differences in keratin association between WT and mutant proteins, may have gone unnoticed.

One strategy to solve this is to express WT and phosphorylation mutant 16E1^E4s using a pIRES-GFP (internal ribosomal entry site – green fluorescent protein) vector. This system would allow translation of 16E1^E4 but also allow GFP translation from the same mRNA. Therefore, any cell that expresses GFP should also be expressing 16E1^E4. The percentage of GFP-positive cells that have visible 16E1^E4, can be counted. These cells would be the ones that are likely to contain keratin-associated 16E1^E4. If WT, T57A and T57D are compared in this way, differences in visible 16E1^E4 and hence differences in keratin-binding, can be deduced. This work has been started by Alan Kennedy (NIMR).

Another approach is to fluorescently tag WT and T57A 16E1^E4, but use different fluorescent proteins (e.g. DsRed, a red fluorescent protein, and GFP) for each type so that they can be coexpressed and distinguished from each other. When expressed separately, they may both bind keratin, as the 16E1^E4 proteins expressed from pMV11 did in this study. However, when coexpressed, WT 16E1^E4 (which should be phosphorylated) may show greater affinity for keratins and therefore occupy keratin binding sites more readily than the T57A protein does. This system may therefore help to visualise whether keratin preferentially binds to T57-phosphorylated 16E1^E4. A similar method was used by Bryan et al. (2000) to show that the MAPK mutant of 11E1^E4 does not colocalise with filaments or with WT 11E1^E4.

6.3.2 To clarify the role of 16E1^E4 threonine 57 phosphorylation in cyclin colocalisation

In this study, the T57A mutant colocalised with cyclin A less frequently than the WT protein did (mean of 44 % and 82 % respectively). This suggests that T57A has a lower affinity for cyclin A, either because of the lack of phosphorylation or the absence of threonine. A future experiment should be the analysis of colocalisation of T57D with cyclin A, to help determine if the difference is due to phosphorylation. Kinase

assays showed that WT, T57A and T57D are phosphorylated at a similar rate by CDK2/cyclin A, but the degree of phosphorylation may not reflect the degree of binding between the 16E1^E4 proteins and cyclin A. To determine if there are differences in binding affinity to cyclin A, tagged (e.g. with GST or histidine), bacterially expressed WT, T57A and T57D could be compared for their ability to pull-down cyclin A from cell extracts. Alternatively, cyclin A could be immunoprecipitated from cells to see if it preferentially co-immunoprecipitates threonine 57-phosphorylated 16E1^E4. It would also be important to determine whether threonine 57 is also important for cyclin B binding.

6.3.3 To further investigate CDK1/2 phosphorylation of 16E1^E4

The role of serine 32 phosphorylation by CDK1/2 is unknown. Considering the importance of CDK/cyclin binding of 16E1^E4 in cell cycle arrest, the effects of serine 32 phosphorylation on cyclin A/B colocalisation and cell cycle arrest should be investigated.

An interesting investigation would be to find whether both CDK1 and CDK2 phosphorylate 16E1^E4 *in vivo* and whether 16E1^E4 is preferentially phosphorylated by either. Instead of using kinase inhibitors which often have non-specific effects (see 4.9.1), another approach is to use engineered kinases that use specific ATP analogues. Such kinases are mutated at their ATP binding site, so that they no longer accept normal ATP but bind an ATP analogue, N^6-benzyl-substituted ATP. Therefore, after incubating a cell extract with the mutant kinase and the radioactive ATP analogue, any radiolabelled protein must be a specific substrate of this kinase. This technique was used to show that a Kaposi's sarcoma-associated herpesvirus (KSHV) protein, K-bZIP, is phosphorylated by CDK1, and to a lesser degree by CDK2 (Polson et al., 2001). These CDKs were mutated to substitute phenylalanine 80 with glycine (F80G), the mutation being homologous to that used in v-src (a tyrosine kinase), the first kinase to be engineered in this way.

6.3.4 To find the 16E1^E4 *in vitro* PKC phosphorylation site and investigate PKC phosphorylation *in vivo*

By MALDI, the *in vitro* PKCα phosphorylation site in 16E1^E4 was mapped to a

region spanning amino acids 35 and 59. Serines 43 and 44 fall in PKC consensus sites, but the S43/44A mutant was still phosphorylated *in vitro* by PKCα (Figure 3.13b). To map the exact phosphorylation site, the other serine or threonine mutants of this region (S49A, T51A, T54A, T57A, T54/57A, T51/54/57A) need to be tested for phosphorylation *in vitro,* although threonine 57 is unlikely to be involved because its phosphorylation causes a gel-shift.

Once the site is mapped, the PKCα phosphorylation mutant can be expressed in cells and a number of properties analysed (for example, its solubility, keratin or cyclin binding, and its ability to cause cell cycle arrest). Phosphospecific antibodies could also be produced for further investigations, especially for the probing of infected lesions or rafts.

6.3.5 To find the effects of 16E1^E4 phosphorylation on the virus life cycle

To find the ultimate effects of phosphorylation on the HPV16 life cycle, studies should be conducted using epithelial systems, either real, infected lesions, or HPV16-containing organotypic raft cultures. For both systems, the use of phosphospecific antibodies would provide valuable information on the distribution and abundance of different phosphorylated or unphosphorylated forms of 16E1^E4. Phosphospecific antibodies are currently being produced, specific for serine 32 and threonine 57 phosphorylation (Appendix.5). This, together with the immunostaining of epithelial protein kinases (e.g. MAPK, PKC, CDK1/2 and PKA) would give insights into the functional role of different 16E1^E4 phosphorylation sites and the kinases involved.

To assess the functional roles more thoroughly, HPV16 genomes mutated to be deficient in a 16E1^E4 phosphorylation event or mimic a phosphorylation event, could be studied in raft culture. Genomes, mutated for serine 32 phosphorylation or threonine 57 phosphorylation, are being produced by Alan Kennedy (NIMR). These will be transfected into keratinocytes from which raft cultures will be grown.

6.4 The targeting of kinases in cancer therapy

Components of the cell signalling pathways are often overexpressed or constitutively active in cancer. This leads to aberrant cell signalling and therefore, uncontrolled growth, survival and metastasis. An increasingly popular method of cancer therapy is to target protein kinases to prevent disregulated proliferation and instead to promote growth arrest or apoptosis.

The EGFR is frequently overexpressed in epithelial cancers (Salomon et al., 1995). The development of reagents that can prevent EGFR activation, for example by inhibiting ligand binding or EGFR tyrosine kinase activity, is in high demand. One example of such a drug is Herceptin®, a humanised monoclonal antibody directed against a member of the EGFR family, HER-2 (Carter et al., 1992; Cobleigh et al., 1999). Herceptin® is being used to treat breast cancer and has received wide publicity. Gefitinib (Iressa) is another agent that inhibits EGFR activation and its mechanism is to block the tyrosine kinase activity (Barker et al., 2001). This drug is still being tested in trials but has been approved for use in advanced non-small-cell lung carcinoma in some countries.

Downstream of EGFR signalling, is the raf/MEK/MAPK pathway, for which a range of inhibitors are being tested. The raf genes are commonly overexpressed and overactive in cancers, for example b-raf has activating mutations in 70 % of malignant melanomas and 15 % of colon cancers (Davies et al., 2002). One raf inhibitor that is in clinical development, is BAY 43-9006 (Sorafenib), which has shown antitumour activity against a range of cancer types including renal cell cancers and colorectal cancers (Wilhelm et al., 2004). MEK inhibitors are also being investigated for their performance in clinical trials. Examples include CI-1040 (Rinehart et al., 2004), PD-0325901 (Lorusso et al., 2005) and ARRY-142886/AZD6244 (Wallace et al., 2004).

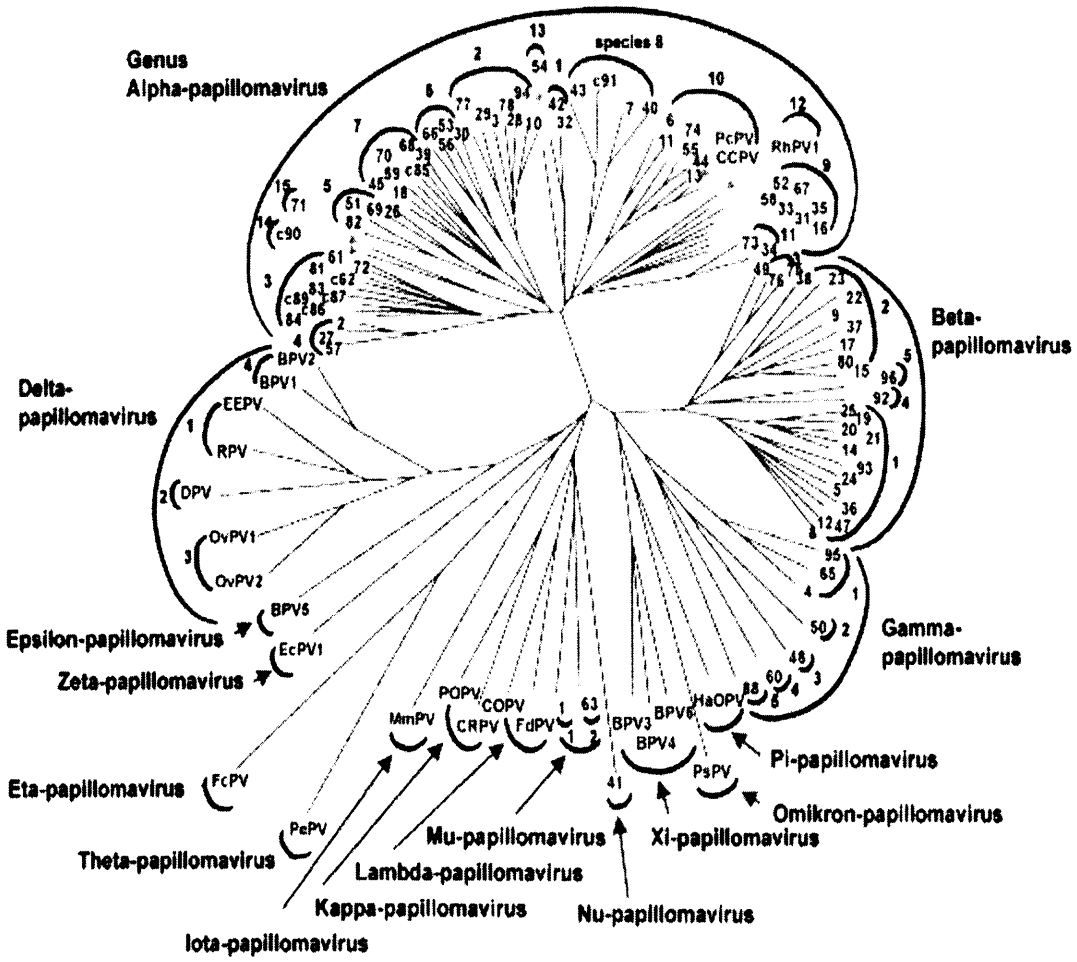
Another type of kinase that can be inhibited for cancer therapy is the CDK family. CDKs are required for cell cycling so blocking their action in malignant cancers would be very effective. Flavopiridol (Kaur et al., 1992; Shapiro, 2004) and Roscovitine (McClue et al., 2002) both inhibit CDK by preventing ATP binding and both have shown promise in clinical trials to date.

The PKC isoforms have roles in cellular proliferation, differentiation and apoptosis, so these kinases have also been targeted for cancer treatment. However, the expression profiles and functions of specific isoforms vary according to the type of cancer and the tissue involved, so targeting PKC is not so straightforward. For example, PKCα is frequently overexpressed in bladder and prostate cancers but downregulated in breast and liver cancers, whereas PKCδ is upregulated in liver cancers and downregulated in bladder cancers (Koivunen et al., 2005). As a result, therapeutics that target PKC molecules are being designed to target specific PKC isoforms. For example, ISIS 3521 (Aprinocarsen) is an oligonucleotide that can bind PKCα mRNA so prevent expression of the kinase and is currently being tested in trials (Advani et al., 2004; Dean and McKay, 1994).

As well as kinase inhibitors, kinase activators can also play a role in cancer therapies, for example, a PKCδ activator, PEP500, has been shown to induce apoptosis in leukaemia cell lines (Hampson et al., 2005). Similarly, Gemcitabine (a pyrimidine analogue that is incorporated into DNA and blocks DNA synthesis), which is widely used in chemotherapy, appears to depend on p38MAPK activation for its apoptotic effects (Habiro et al., 2004).

Considering the extent to which the HPV life cycle and protein function is dependent on signalling pathways, the targeting of kinases in the treatment of HPV infections or HPV-induced cancers, is an attractive approach. For example, if E1^E4 activity can be shown to be enhanced *in vivo* by modulators of kinase activity, HPV infections could be directed towards late viral events instead of cancer. For this technology to be developed in the future, the regulation of virus protein function by protein kinases must continue to be investigated.

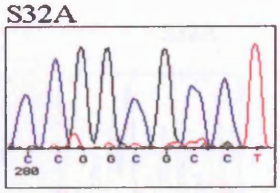
Appendix.1 Papillomavirus phylogenetic tree



The papillomavirus phylogenetic tree taken from de Villiers et al. (2004). The outermost semi-circles indicate the papillomavirus genus and the number at the inner semi-circles refers to the papillomavirus species. The numbers at the ends of branches identifies the HPV type, 'c' refers to candidate HPVs and abbreviations are names of animal papillomaviruses.

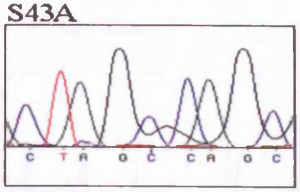
Appendix.2 Sequencing results for His-E1^E4 mutants

S32A



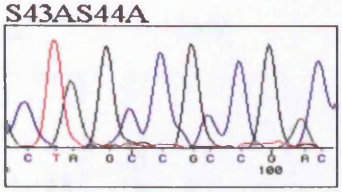
P A P

S43A



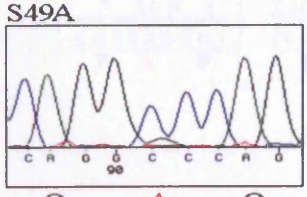
L A S

S43AS44A



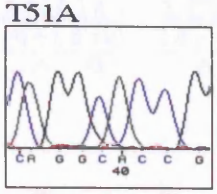
L A A D

S49A



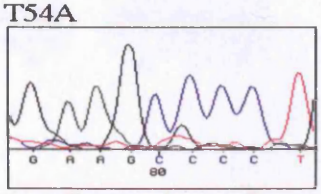
Q A Q

T51A



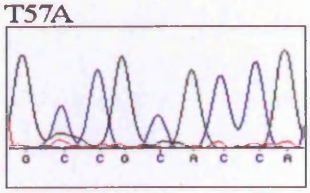
Q A P

T54A



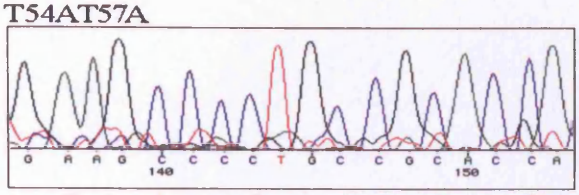
E A P

T57A



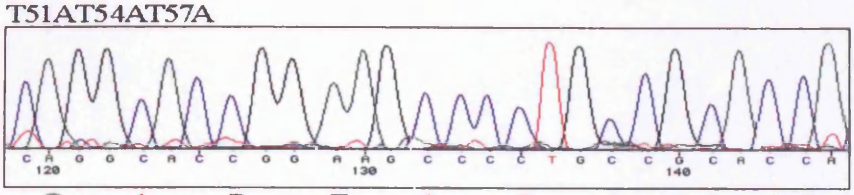
A A P

T54AT57A



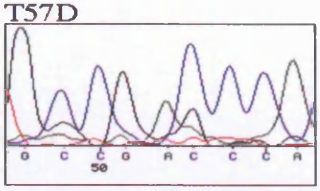
E A P A A P

T51AT54AT57A



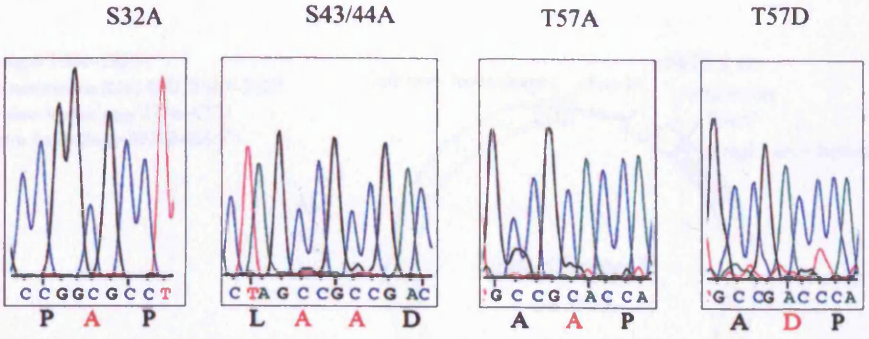
Q A P E A P A A P

T57D

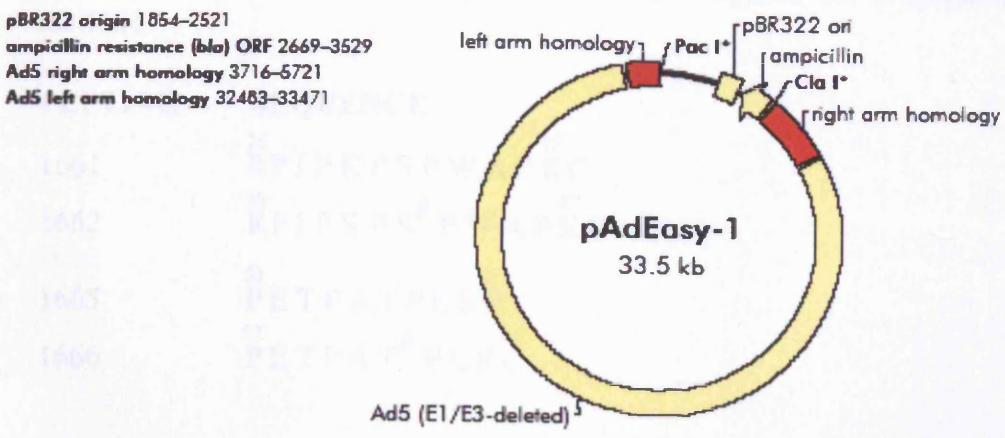


A D P

Appendix.3 Sequencing results for pMV11 E1^E4 mutants



Appendix.4 pAdEasy-1 vector map



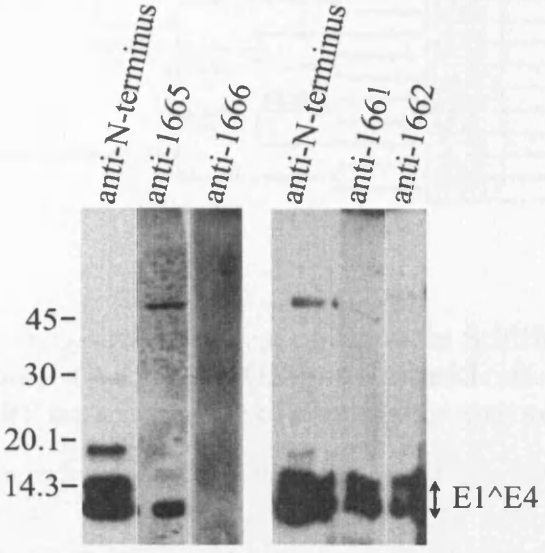
Plasmid map indicates that the adenoviral genes, E1 and E3, are deleted (*taken from* http://www.stratagene.com/products/displayProduct.aspx?pid=86).

Appendix.5 Phosphospecific antibody production

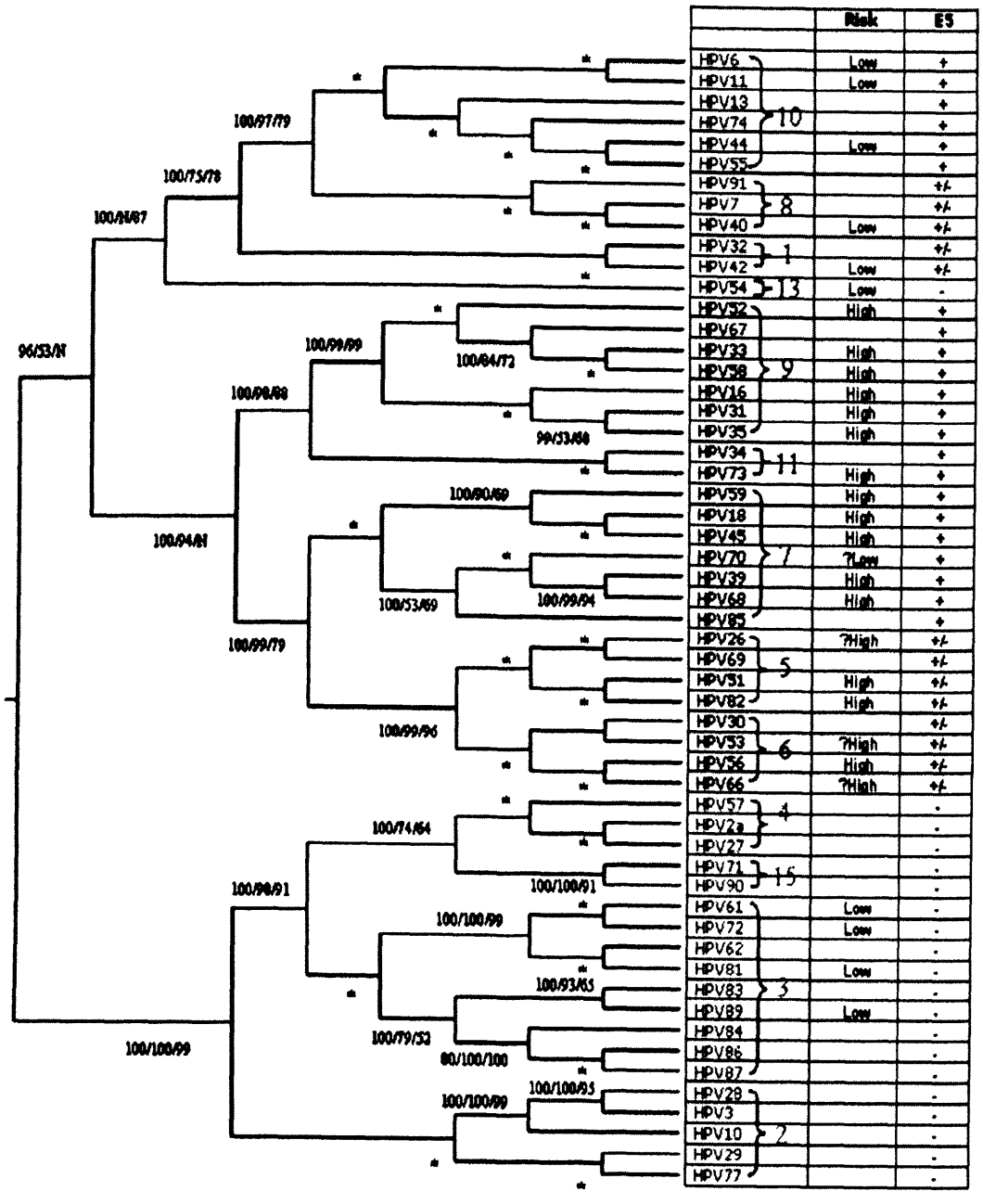
The peptides, 1661, 1662, 1665 and 1666 were synthesised, conjugated to mcKLH and used to immunise rabbits. Their sequences are shown below. 1661 and 1662 were designed to be specific for unphosphorylated and phosphorylated S32 respectively, and 1665 and 1666 were designed to be specific for unphosphorylated and phosphorylated T57 respectively. 1661 and 1662 peptides contained a cysteine residue at their C-terminus which is not part of the 16E1^E4 sequence, but was required for conjugation to mcKLH.

PEPTIDE	SEQUENCE
1661	^{26}RPIPKPSPWAPK^{37}C
1662	^{26}RPIPKPS^{P}PWAPK^{37}C
1665	^{52}PETPATPLSC^{61}
1666	^{52}PETPAT^{P}PLSC^{61}

After the immunisation programme, the unpurified terminal bleeds were used in Western blots (at a 1:250 dilution) to test for their reactivity against a rAdE1^E4-infected SiHa cell extract. The anti-N-terminus rabbit polyclonal was also used for comparison. Anti-1661, -1662 and -1665 appeared immuno-reactive to E1^E4, but anti-1666 did not detect the protein. These antibodies need to be purified using peptide affinity purification to produce anti-E1^E4 antibodies specific for unphosphorylated



Appendix.6 Phylogenetic analysis of anogenital HPVs



Phylogenetic analysis of anogenital HPV types taken from Schiffman et al. (2005). The presence (+) or absence (-) of an E5 ORF, is indicated. +/- is used to depict the presence of an E5 ORF but the absence of a translation start codon.

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